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13. ABSTRACT *(Maximum 200 Words)*

Ovariectomized, adult rats, were injected with the tumor initiator methylnitrosourea (MNU). Tumor incidence without hormone replacement was 3%. Continuous estradiol or estrone replacement for 8 months yielded 24% or 54% tumor incidence, respectively. When progesterone was included with estrone, tumor incidence was 88%, significantly greater ($p = 0.042$) than the incidence with estrone alone. Thus, progesterone synergizes with estrone to induce tumorigenesis. Synergistic stimulation of glandular growth was associated with this increase in tumor incidence.

Ovariectomized mice were treated with ß-hexachlorocyclohexane or o,p'-DDT by continuous, slow release capsule; the lowest blood levels required to produce an estrogenic effect were 42 ng/ml or 18 ng/ml, respectively. These concentrations are only slightly higher than those found in humans, suggesting that these compounds may pose a risk at current exposure levels.

Transfection studies using mutant forms of estrogen receptor and a luciferase reporter expression vector showed that the two activation domains within the receptor, AF-1 and AF-2 are required for full activity of xenoestrogens. With wildtype receptor, xenoestrogens were as effective as estradiol. When either activation domain was mutated, the xenoestrogens were 33-50% less effective than estradiol. Thus, xenoestrogens depend on the combined effects of AF-1 and AF-2 for their full effect.

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I. INTRODUCTION

Tumorigenesis is classically divided into two steps, initiation and promotion. Initiation is likely to be the result of some genotoxic insult. Tumor promotion depends on increased growth of the initiated cell and it may be the result of another genotoxic insult to growth regulatory machinery or it may derive from epigenetic stimuli that are normally involved in regulating growth of the original target tissue. Because of its role in mammary gland development, estrogen has long been suspected to behave as a tumor promoter of mammary cancer. This notion is supported by the fact that mammary tumor growth may be inhibited by antiestrogens. It follows that xenoestrogens could supply tumor promoter activity to initiated mammary cells. The studies devised under this grant were designed to examine the tumor promoter activity of two xenoestrogens, o,p'-DDT and ß-hexachlorocyclohexane (ß-HCH), to define the role of fat stores of these compounds in mammary tumorigenesis, and to determine the molecular mechanism of estrogenic action of o,p'-DDT and ß-HCH. Four model systems were used: 1) A rat model of mammary tumorigenesis used methylnitrosourea as the tumor initiator factor, that by itself is without tumor promoter activity. The model was manipulated in a way to allow testing of tumor promoter activity of estrogens, both natural or synthetic. 2) A mouse xenograft model, in which human cancer cells were grown as tumors, was also used to determine the tumor growth stimulatory activity of estrogenic compounds. 3) The ovariectomized mouse served as a means of testing the relationship between blood levels and estrogenic responses in the classical target tissues of the uterus and vagina. 4) Cell cultures were used to test the molecular structure of the estrogen receptor that mediates the effects of xenoestrogens.

The body of this report will be divided into sections according to research models studied; there will be one additional section describing work that was either indirectly supported by the grant or which stemmed from the work in the S.O.W. but was unrelated to breast cancer. The Results and Discussion subsection of each report will deal with the scientific significance of the observations made to date, while a separate section will be allotted to the discussion of the relevance to the original Statement of Work.

II. BODY OF REPORT

1. MNU-INDUCED MAMMARY TUMORS IN THE RAT

a. Background & Introduction

MNU induces estrogen-dependent mammary tumors in the rat and this has become a standard model system for testing tumor growth inhibiting activity of potential estrogen antagonists. Since injected MNU is fully oxidized and disappears from the animal's system within a matter of less than one hour (1-3), it must produce its tumor initiating effects within minutes of administration. Fully mature, young rats (age, 49-56 days) have been shown to respond optimally to the tumorigenic effect of MNU. Estrogen-dependence of MNU-induced tumors is demonstrable by the following observations: ovariectomy or antiestrogen treatment performed after MNU initiation blocks tumor development; ovariectomy leads to tumor stasis or shrinkage and estrogen replacement will cause resumption of tumor growth. In other model systems the xenoestrogens, o,p'-DDT and ß-HCH have been shown to support growth of experimental mammary tumors and/or human breast cancer cells in culture (4, 5). These observations suggest that estrogen is the ovarian factor that is required for tumor progression and that xenoestrogens can substitute for the natural hormone in this regard. However, the tumor promoter activity of estrogens has not been tested directly in the standard MNU rat model.

A study was designed to address the question of whether estrogen behaves as a tumor promoter in the MNU-initiated rat model. There is only one prior report in which MNU-treated animals were ovariectomized before detectable tumors were formed and then treated with estrogen to promote tumorigenesis (6). That study was performed in an unorthodox protocol in which neonatal (2-day-old) rats were treated with MNU, followed by tamoxifen treatment until 30 days of age, at which time animals were ovariectomized, and then estrone treatment was begun at 3.5 months of age. In this protocol, 50% (6/12) estrogen reconstituted animals developed tumors compared to rates of 80% (35/44) in ovary-intact control animals and 4% (1/27) in ovariectomized, untreated, MNU-initiated rats. Thus, from this single study, it would appear that estrogen can act as tumor promoter in an MNU-initiated mammary gland and therefore estrogen-replacement in ovariectomized MNU-initiated animals should serve as the positive control for the xenoestrogen studies.

In our study, animals (49-56 days old) were ovariectomized at the time of MNU treatment and either supplied with a continuous release estrogen capsule or received no further treatment. The notion that estrogen serves a tumor promoter in the mammary gland was directly tested in this manner. In addition, this model was used to test of whether xenoestrogen treatment of ovariectomized rats would be sufficient to insure tumor progression. The experiments completed to date tested the tumor promoter activity of estradiol and estrone, the latter being a weaker estrogen that predominates in postmenopausal women and which may be more comparable to the weakly estrogenic xenobiotics. Our earlier data indicated that animals supplemented with estradiol had a tumor incidence of only 24% while those treated with estrone had a 55% tumor incidence. Thus, there may be a fundamental difference in the tumor promoter activitys of these two estrogens, and neither alone is sufficient to reproduce the effect of

the ovary. Further experiments were performed in which estrone and progesterone were administered in an attempt to achieve full tumor promoter activity.

Others had reported that high doses of estrogen, with or without progesterone, could actually protect against MNU-induced tumorigenesis (7). It may be that the estrogen treatments applied in our experiments are also exerting a degree of protection, and that the final tumor incidence is a matter of balance between tumor promoter and anti-tumor action of the estrogen. Thus, groups of MNU-treated, ovary-intact animals were treated with estradiol or estrone.

b. Experimental Procedures and Results

The Methods and outcome of the basic studies are reported in a manuscript that has been submitted for publication in Carcinogenesis. This manuscript is attached as an appendix item.

Briefly, rats (49-56 days old) were ovariectomized, treated with MNU intravenously, and with test compound (estradiol, estrone, progesterone, ß-HCH) by slow release capsule implanted subcutaneously; control animals received and empty treatment capsule. Additional animals were treated with empty capsules or ß-HCH and were subjected to dietary restriction (40% of usual intake) for 1 out every 3 weeks.

The data reported show that estrone is a better tumor promoter than estradiol. Tumor incidence after 8 months was 54% and 24% for estrone and estradiol treatments, respectively, compared to 0-3% in controls from two separate experiments. Progesterone administered alone had no effect, no tumors formed in the 15 treated animals. On the other hand, progesterone synergized with estrone to produce an incidence of 88% at 8 months.

Treatment with ß-HCH, with or without dietary restriction did not produce tumors in ovariectomized, MNU initiated rats. Yet to be determine is whether this weak estrogen might be effective in a hormonal background dominated by progesterone. Unfortunately, budgetary constraints of the grant did not allow for continuation of this work.

As mentioned earlier, estrogen administered to ovary intact rats will suppress tumorigenesis induced by MNU. We treated intact 56-day-old rats with MNU and implanted capsules of estradiol, estrone, or empty capsules. Tumor incidence was 96% in intact controls and it was reduced to 56% or 19% by treatment with estrone or estradiol, respectively (see manuscript, Fig. 2). Thus, estradiol was better than estrone at inhibiting tumorigenesis under these conditions.

Examination of mammary glands by whole mount preparation showed that one month of hormone treatment induced a growth response in the tissue (see manuscript, Figs. 3 and 4). Mammary glands of intact control animals had an abundance of end buds decorating the ducts, while the ducts of ovariectomized animals were straight and had very few end buds. Treatment with estrone or estradiol induced growth of the mammary tree such that the ducts were decorated with end buds, but not to the extent of the normal gland. Progesterone alone had no effect on mammary gland growth.

The combination of progesterone and estrone produced an even greater growth response.

Blood estrogen levels were analyzed using a radioimmunoassay for estradiol; in the manner used, the assay measures both estrone and estradiol. In estrone-treated animals, estrogen levels reached a peak of 60-80 pg/ml during the first week, returning to 40 pg/ml by one month. We had previously reported that the estrogen concentrations were 18-24 pg/ml after 8 months of treatment (previous reports).

The biological implications of these observations are discussed in the manuscript. Clearly there are difference in the tumor promoter and tumor inhibitory actions of estradiol and estrone. Such differences have clinical implications as well. It may be possible to develop a selective estrogen receptor modulator (SERM) with enhanced tumor inhibitory activity and diminished tumor promoter activity. In addition, the synergy between estrone and progesterone seen in this experimental system supports the notion that combined hormone replacement therapy might pose a greater risk for breast cancer than estrogen replacement therapy. Two recent publications have indicated that this may indeed be the case (see manuscript references 27 and 28).

2. MOUSE MODEL – MCF-7 XENOGRAFTS

a. Background & Introduction

The xenograft of human tumor cells in athymic mice has become another standard model for testing the tumor growth promoting or inhibiting effects of a test compound. We had shown earlier that ß-HCH stimulated growth of tumors produced from the human breast cancer cell line, MCF-7 (5). In that study, host animals were treated with a single Silastic capsule containing either ß-HCH or estradiol; tumor growth was essentially equivalent for both treatments over a 16 day period.

In three experimental attempts these observations could not be reproduced. The tumors produced exhibited a much slower growth rate than earlier and they were not at all responsive to xenoestrogen.

b. Methods

MCF-7 Xenograft Experiment

Two separate experiments were performed. In each case host animals (5-6 week old, athymic, female Balb/c mice) were implanted with tumor that was derived from tumors passaged in athymic mice. The original tumor was produced by injection 10^7 MCF-7 cells in Matrigel subcutaneously, as described in the previous report. Once the resultant tumor reached 1.5 cm diameter, the host was killed and the tumor was excised. Tumor tissue was minced and passed through an 40 guage stainless steel mesh (Cellector) and suspended in culture medium (DMEM). The tumor tissue was washed by allowing the fragments to settle out of suspension, removing and replacing the medium. A final suspension was made to 50% (vol/vol) and this was used for injection. Each host animal received two subcutaneous injections (100 μl) of tumor slurry through an 18 guage needle.

In the first experiment, a single Silastic capsule containing estradiol was inserted subcutaneously at the time of tumor implantation. After 5 weeks of tumor growth, the estradiol capsules were removed from 28 animals and 16 of these were treated by inserting 4 capsules of ß-HCH into each animal, the other 12 animals were left untreated. There were 6 animals in which the original E2 capsule remained; these served as positive controls. After one additional week, 8 of the ß-HCH-treated animals and 6 of the untreated animals were placed on periodic food restriction; they received 50% of the normal food intake for 3 days of each week. Tumors were measured with calipers and tumor volumes were determined, as described earlier.

In experiment no. 2, the tumor used was derived from a tumor that grew slowly in an intact athymic host without added E2. It was hoped that this tumor would prove more sensitive to weak estrogens. This tumor was transplanted to experimental host animals and each host was given three daily injections of estradiol (10 μg/kg/day) to insure tumor take and initial growth. Three weeks after the last E2 injection, groups of animals received a single capsule of E2, 4 capsules of o,p'-DDT, or 4 capsules of ß-HCH and tumor growth was followed on a weekly basis; animals that received no further treatment after the initial injections served as negative controls. Tumor volume was measured on a weekly basis.

c. Results and Discussion

The original observations of xenoestrogen-induced tumor growth could not be reproduced. The tumors produced exhibited a much slower growth rate than earlier and they were not at all responsive to xenoestrogen. Figures 2.1 and 2.2 show the growth responses in two separate experiments. In the first experiment, tumor was initially stimulated by implantation of an estradiol capsule. Experimental treatments began with removal of the E2 capsule after 5 weeks of growth; at this time the average tumor volume was equal to 570 mm^3. In this experiment, ß-HCH did not substitue for E2 (Fig. 2.1); furthermore, fasting had no effect on tumor growth.

In the second experiment the implanted tumor tissue came from a tumor that had grown in an intact female host without steroid supplementation. In this case the tumor volume at the beginning of the experimental treatments was much smaller, with an average of 45 mm^3. These tumors grew under estradiol and o,p'-DDT stimulation; ß-HCH produced only a small growth effect (Fig. 2.2). This is in contrast to the original experiments which indicated that ß-HCH was as active as estradiol in this growth assay (5).

The reasons for the inconsistencies in this tumor growth assay are unknown. Perhaps there has been a subtle change in the character of the MCF-7 cells used. Unfortunately, due to an accident in the lab we have lost all of the early passages of the MCF-7 cell stock. Clearly, the original tumors grew at a much faster rate and they continued growth for several days without estradiol supplementation (5). Also, the size of the tumor at the initiation of experimental treatment may be very important; here, the very small tumors responded to o,p'-DDT and ß-HCH while the tumors that were large at the outset did not respond.

3. OVARIECTOMIZED MOUSE MODEL – UTERINE & VAGINAL EPITHELIAL BIOASSAYS

a. Background & Introduction

Several organochlorine xenobiotics, such as ß-HCH and o,p'-DDT, were previously determined to be estrogenic using standard rodent bioassay systems. Typically, uterine weight increases or vaginal epithelial cornification were used as the endpoints in short term experiments following a single bolus injection of 100-500 mg/kg of test compound. On the other hand, blood levels of organochlorine compounds in humans are typically in the ng/ml range. These observations suggest that because organochlorine xenoestrogens are so weak in vivo, they pose little risk. However, humans (and wildlife) are exposed to these low levels of compound continuously rather than the single episodic rise and fall of systemic concentrations that follows a single injection in the experimental animal. Therefore we tested the estrogenic responses in ovariectomized mice that were chronically exposed to low levels of ß-HCH or o,p;'-DDT and we correlated the blood levels achieved during this exposure to the degree of estrogenic response. In this way we hope to better model the human situation and to determine if the low blood concentrations (ng/ml range) might pose a risk due estrogenic activity.

b. Methods/Results/Discussion

The results of this study were reported in the publication: EM Ulrich, A Caperell-Grant, S-H Jung, RA Hites, RM Bigsby Environmentally relevant xenoestrogen tissue concentrations correlated to biological response in mice. Environ Health Perspect 108:973-7, 2000 (included in appendix). This report represents a major portion of the work completed by Elin Ulrich in fulfillment of the requirements for her doctoral degree, which was granted August, 2000.

Brielfy, Silastic capsules containing ß-HCH or o,p'-DDT were implanted sc in ovariectomized mice. Controls consisted of animals receiving an empty implant or one containing estrone. After one week animals were killed, exsanguinated and their uterine and vaginal tissues prepared for histological analysis. Uterine epithelial cell height and vaginal epithelial thickness were used as estrogenic endpoints. Blood serum concentration of test compound were measured by GC-mass spectrometry.

The results showed that blood levels as low as 18 ng/ml o,p'-DDT and 42 ng/ml ß-HCH were associated with a significant increase in the estrogenic parameters. Thus, the ng/ml range of organochlorine compounds found in human blood may indeed pose a

4. ESTROGEN RECEPTOR-REPORTER GENE TRANSFECTION STUDIES

a. Background & Introduction

[This material is presently "in preparation" for submission to a peer-reviewed journal for publication.]

Estrogens produce a variety of biological effects in different tissues: they stimulate growth of the uterus and mammary gland cells, maintain bone mineral density, and reduce serum LDL (8, 9). Recently, it has been appreciated that certain compounds selectively modulate these biological effects; for instance, tamoxifen behaves as an antiestrogen in mammary carcinoma cells but is stimulatory in the uterus and bone (9). The divergent effects of compounds such as tamoxifen has led to the hypothesis that there are substance that can act as selective estrogen response modulators (SERMs) (9). Some xenoestrogens appear to behave as SERMs, modifying biological responses in some tissues but not in others (8). The mechanisms by which SERMs specify tissue response is unknown.

The estrogen receptor protein can be divided into four functional, and physically distinct, domains: ligand binding domain; the DNA binding domain; the N-terminal transcriptional activation function domain (AF-1); and the C-terminal transcriptional activation function domain (AF-2) (10 - 13). AF-1 and AF-2 behave differently depending the ligand present, the cellular context, and the gene promoter under activation (13, 14). It may be that additive or synergistic effects of the two activation domains are regulated differentially by SERMs.

Steroid receptors do not activate gene transcription on their own, rather they elicit this function by binding coactivator proteins and thereby generating and interacting with a large complex of transcription factors (15). The coactivator proteins, SRC-1, TIF2 and GRIP-1, interact with the estrogen receptor mainly at the AF-2 region (15-18). However, recently it has been shown that coactivators can act through the AF-1 region as well (19, 20). This interaction at two sites within the protein may allow for the additive or synergistic actions required to explain the function of SERMs.

The three-dimensional structure of the estrogen receptor has been partially deciphered by examining the crystal structure of the carboxy half of the protein in the presence or absence of ligand (21, 22). It was found that compounds with antiestrogen activity, such as tamoxifen and raloxifene, produce a conformation of receptor that would preclude its interaction with coactivator in the AF-2 region, while estradiol produces a conformation that promotes this interaction (21, 22). Although this suggests a mechanism for the antiestrogenic effects of tamoxifen or raloxifene, it does not explain how they can behave as estrogen agonists in certain tissues.

The present studies were designed to dissect the ability of xenoestrogens to function through the different domains of the estrogen receptor. Mutant receptors lacking either AF-1 or AF-2 were used in transfection assays and the transactivational effects of the compounds were compared against that of estradiol. The results suggest that xenoestrogens are more dependent upon the interaction of the two functional domains than is estradiol and that this is especially true for particular compounds.

b. Materials and Methods

Reagents: Cell culture reagents (phenol-red free MEM, glutamine, phosphate buffered saline calcium and magnesium free, and DMRIE-C) were purchased from Life Technologies. Fetal calf serum and charcoal-stripped fetal calf serum were purchased

from Hyclone Laboratories. Estrogen, bisphenol A, and octylphenol were purchased from Sigma. ß-HCH and o,p'-DDT and were purchased from Chemservice. Luciferase assay reagents were purchased from Promega while the ß-galactosidase assay reagents were purchased from Tropix.

Plasmid construction: Wild-type ERα expression plasmid ERαpCMV5 (23), parental pCMV5 plasmid, R507L (24), S118A (25), and ERαΔAB (25) were provided by Benita Katzenellenbogen. Mutant ERα expression plasmids HE11 and HE48 (10) were provided by Pierre Chambon. Mutant ERα expression plasmids TAF1 and ER-Null (13) were provided by Donald McDonnell. The ß-galactosidase expression plasmid pAD was purchased from Stratagene. The parental luciferase reporter plasmid pGL3 was purchased from Promega.

The reporter plasmid was constructed as follows. First, the minimal promoter region of the pS2 gene (10, 26, 27) was synthesized by hybridizing the oligonucleotides:

GATCCCCGGGCCTCCTTAGGCAAATGTTATCTAACGCTCTTTAAGCAAACAGAGCC
TGCCCTATAAAATCCGGGGCTCGGGCGGCCTCTCATCCCTGACTTCGA

and

AGTCAGGGATGAGAGGCCGCCCGAGCCCCGGATTTTATAGGGCAGGCTCTGTTTGCT
TAAAGAGCGTAGATAACATTTGCCTAAGGAGGCCCGGG.

The DNA was blunted with Klenow fragment and ligated into the blunted HindIII site of pGL3; this plasmid is referred to as pS2Luc. To make this reporter estrogen responsive, we synthesized an enhancer region containing two estrogen response elements (EREs, underlined in the sequence below) by hybridizing the following oligonucleotides:

C<u>AGGTCACAGTGACCT</u>GATCAGCTAGTC<u>AGGTCACAGTGA</u>CCTTCGTAC

and

GAAGGTCACTGTGACCTGACTAGCTGATCAGGTCACTGTGACCTGGTAC.

This DNA was blunted with Klenow fragment and ligated into the blunted KpnI site of pS2Luc to make ERE2pS2Luc.

The estrogen receptor mutant containing an AF-1 deletion, ERαΔ2-150, was constructed by amplifying a PCR fragment from ERαpCMV5 under the manufacturers suggested conditions using primers: GCCCAGGGGCCGCTCCAGGGGGATC and CATGGTCCGTGGCCGCGGGCAGGGT and r*Tth* DNA polymerase XL (Perkin-Elmer). The PCR fragment was circularized with T4 ligase (Gibco) and treated with DpnI. Then, the DNA was used to transform DH5α bacterial cells. The ERE2pS2Luc and ERαΔ2-150 plasmids were confirmed by dideoxysequencing.

Cell culture andTransfection: MDA-MB-231 breast cancer cells (ATCC) were maintained in phenol-red free MEM containing 2 mM glutamine and 5% fetal calf serum. In preparation for transfection, cells were plated at a density of 200,000 cells per 35 mm well in phenol-red free MEM containing 2 mM glutamine and 5% charcoal-stripped fetal calf serum. The next day cells were transiently transfected using DNA-DMRIE-C lipid complexes. Briefly, 500 ng of ERE2pS2Luc reporter plasmid, 100 ng of pAD ß-galactosidase expression plasmid, and 25 ng estrogen receptor expression plasmid were incubated for 30 minutes with 1 ml of phenol-red free MEM and 4 μl of DMRIE-C

reagent. DNA-lipid complexes (1 ml) were placed on each well of MDA-MB-231 cells for 6 h.

The transfected cells were treated with various concentrations of estradiol, bisphenol A, octylphenol, o,p'-DDT, ß-HCH, and DMSO for 24h in medium supplemented with 5% charcoal-stripped serum. Cells were washed once with phosphate buffered saline calcium and magnesium free and lysed with 200 μl of reporter lysis buffer (Promega). Lysate was exposed to one freeze-thaw cycle and then centrifuged. Briefly, 80 μl of luciferase reagent was mixed with 20 μl of lysate and light output was measured with a ML3000 luminometer. A separate 20 μl sample was analyzed for ß-galactosidase activity by mixing 80 μl of assay buffer and incubating at room temperature for 1 h. Then, 100 μl of light accelerator was added and light output was measured. The ß-galactosidase measurements normalized the observed luciferase activity.

c. Results & Discussion

Since certain environmental estrogens show tissue-selective effects in the ovariectomized rat (8), we tested the hypothesis that these compounds produce distinct changes in transcriptional efficiency of the estrogen receptor when compared to estradiol and that the relative contribution of the two activation function domains within the receptor would be ligand specific. To measure the transcriptional effect of these environmental estrogens, we developed a co-transfection assay. The assay allowed us to introduce wild-type estrogen receptor cDNA as well as specific functional mutants of the receptor into an estrogen receptor negative cell line, MDA-MB-231. Using a luciferase reporter plasmid that contained two consensus estrogen response elements upstream of a minimal pS2 promoter, ERE2pS2Luc, we measured the transcriptional activity of the estrogen receptor. The amount of luciferase produced by the transfected MDA-MB-231 cells is dependent on the presence of estrogen receptor and concentration of compound placed on the cells during culture. A hormone-independent ß-galactosidase expression vector was cotransfected into the cells and the ß-galactosidase activity was used to normalize for well-to-well variation in transfection efficiency. Maximal luciferase activity at 10^{-9} M estradiol was defined as 100% activity and was used to determine the relative transcriptional activity of compounds for each estrogen receptor expression plasmid tested.

Wild-type estrogen receptor: To measure the activity of the environmental estrogens, bisphenol A, octylphenol, o,p'-DDT, and ß-HCH, we introduced wild-type estrogen receptor, ERαpCMV5, into MDA-MB-231 cells. With 10^{-10} M estradiol, the luciferase activity reached maximal levels producing a five-fold increase relative to DMSO control (Fig. 4.1). The half-maximal concentration of estradiol (K_d) was approximately $1.0 \bullet 10^{-11}$ M (Fig. 4.1), similar to that found earlier by Reese and Katzenellenbogen (23).

Since our co-transfection assay was sensitive enough to detect transcriptional activation by a subnanomolar concentration of estrogen, we could measure increases in luciferase activity induced by weak binding environmental estrogens. With bisphenol A and octylphenol, the luciferase activity increased about five-fold relative to DMSO control (Fig. 4.1). So the environmental estrogens, bisphenol A and octylphenol, activate the wild-type estrogen receptor with the same efficacy as estradiol. The concentration of bisphenol A and octylphenol required to reach maximal luciferase activity was 10^{-6} M.

The observed K_d for bisphenol A was approximately $0.3 \bullet 10^{-6}$ M whereas the K_d for octylphenol was approximately $1.0 \bullet 10^{-7}$ M (Fig. 4.1). So, although bisphenol A and octylphenol activate the estrogen receptor as strongly as estrogen, the compounds have a four-log reduced potency reflecting their weak binding affinity for the estrogen receptor.

The environmental estrogen, o,p'-DDT, showed an interesting induction of luciferase activity compared to estradiol. The maximal luciferase activity by 10^{-5} M o,p'-DDT was about seven-fold compared to DMSO control (Fig. 4.1). However, the concentration of o,p'-DDT required to reach plateau was not determined because the limits of solubility for o,p'-DDT had been reached at 10^{-5} M. So, the observed maximal transcriptional activity for o,p'-DDT was greater than the transcriptional activity for estradiol. The super-activation of the estrogen receptor by o,p'-DDT may be due to a ligand-independent activation of the estrogen receptor through phosphorylation by kinases activated by high concentrations of o,p'-DDT (28).

β-HCH was also tested in our co-transfection assay. Although β-HCH does not displace tritiated estradiol from receptor (5), it does activate luciferase expression in our system. Luciferase activity is increased about four-fold compared to DMSO control (Fig. 4.1). Again, similar to o,p'-DDT, ß-HCH did not show a plateau of activity at the highest concentration tested. However, unlike o,p'-DDT, ß-HCH did not show a super-activation of the wild-type estrogen receptor (Fig. 4.1).

Estrogen receptor with loss of AF-1 function. To measure the activity of the environmental estrogens on an estrogen receptor without an AF-1 domain, we introduced the ERαΔ2-150 or ERαΔAB plasmids into MDA-MB-231 cells. Although the absolute amount of luciferase expression was reduced with the ER mutants compared to wild-type ER, the fold response remained five- to eight-fold with the mutants tested (Fig 4.2, 4.3). For example, estradiol increased the luciferase activity about five-fold relative to DMSO control (Fig. 4.2, 4.3). Furthermore, the K_d for estradiol using the AF-1 defective mutants was similar to the K_d for the wild-type receptor (Figs. 4.1 - 4.3). The literature shows the binding affinity for AF-1 mutant receptors does not differ from the wild-type receptor. Estradiol produced maximal luciferase activity at 10^{-10} M. Similar to estradiol, bisphenol A, octylphenol, o,p'-DDT, and ß-HCH had K_d's that were identical when comparing between wild-type receptor and the AF-1 defective mutants (Fig 4.2, 4.3). The experiment suggests that deleting the AF-1 domain does not reduce the binding affinity of compounds to the hormone binding domain of the estrogen receptor.

Whereas the environmental estrogens activated the wild-type receptor as efficaciously as estradiol, the AF-1 defective estrogen receptors had reduced transcriptional activity. With bisphenol A, the luciferase activity was only 54% of the maximal activity reached by using 10^{-9} M estradiol (Fig. 4.2). Similarly, with the ERαΔAB mutant treated with bisphenol A, luciferase activity reached 45% of maximal estradiol activity (Fig. 4.3). The transcriptional activity was also reduced for octylphenol, o,p'-DDT, and ß-HCH. Octylphenol showed 65% of the maximal estradiol activity (Fig. 4.2). Also, with the ERαΔAB mutant, octylphenol was 84% of maximal estradiol activity (Fig. 4.3). Like the wild-type estrogen receptor, the transcriptional response of the AF-1 defective mutants was saturated by micromolar concentrations of bisphenol A and octylphenol (Fig. 4.1 – 4.3). In contrast, high concentrations of o,p'-DDT and ß-HCH did not saturate the

transcriptional response of ERαΔ2-150 or ERαΔAB (Figs. 4.1 – 4.3). However, the maximal transcriptional response of these two compounds was reduced in ERαΔ2-150. ß-HCH activated ERαΔ2-150 at 67% of the maximal estradiol activity while o,p'-DDT activated at 92% of the maximal estradiol activity (Fig 4.2). Thus, o,p'-DDT lost its ability to super-activate transcription when the receptor lacked the AF-1. Overall, the transcriptional activity of the AF-1 defective mutants is reduced compared to the wild-type receptor. Moreover, the transcriptional activity induced by environmental estrogens is further reduced compared to estradiol.

The literature shows that the estrogen receptor uses both AF-1 and AF-2 for transcriptional activation (11, 13, 29). Our data agrees with published observations that the reduced transcriptional activity of ERαΔ2-150 using estradiol is due to activity from the functional AF-2 domain. However, since the activity from environmental estrogens is further reduced, our data also suggests that there are different ligand-induced conformations of the AF-2 domain. To increase reporter activity, the AF-2 domain interacts with the transcriptional machinery through coactivators such as the SRC-1 family, GRIP1, and TIF2 (15-18). Because the transcriptional activity is reduced with environmental estrogens, it implies that the interaction of AF-2 with coactivators is reduced. The conformation adopted by the hormone binding domain determines the affinity of the estrogen receptor for coactivators. For example, tamoxifen and raloxifene produce conformations in the carboxy portion of the estrogen receptor that cannot interact with coactivators (21, 22). Perhaps the environmental estrogens cause unique conformations of the estrogen receptor so that the AF-2 binds to coactivators with different affinities. The conclusion would be that the transcriptional machinery senses the conformation of the estrogen receptor bound by environmental estrogens. When operating with an isolated, functioning AF-2, the conformation adopted by the receptor bound to an environmental estrogen shows reduced transcriptional activity.

Estrogen receptor with loss of AF-2 function: To measure the activity of the environmental estrogens on an estrogen receptor without an AF-2 domain, we introduced the TAF1 plasmid into MDA-MB-231 cells. The TAF1 plasmid has point mutation in the AF-2 region of the receptor that interacts with coactivators (13); thus, this mutant receptor depends on the AF-1 domain for transactivational function. Similar to ERαΔ2-150, the absolute amount of luciferase expression was reduced with TAF1 compared to wild-type ER; however, the response at 10^{-9} M estradiol was 10-fold compared to vehicle control (Fig. 4.2). We observed that the dose-response curves were right-shifted one log compared to the wild-type receptor (Figs. 4.1 & 4.4). In contrast, TAF1 has a binding affinity for estradiol similar to wild-type estrogen receptor since the point mutations in TAF1 do not interfere with hormone binding (13). Lower numbers of receptors in the transfected MDA-MB-231 cells may explain the right-shift in the dose-response curves. Other transfection experiments using TAF1 showed dose-response curves similar to wild-type receptor (data not shown).

Each dose-response curve from the environmental estrogens had a one log right-shift when compared to wild-type estrogen receptor (Figs. 4.1 & 4.4). Whereas the compounds activated the wild-type receptor as efficaciously as estradiol, this AF-2 defective estrogen receptor had reduced transcriptional activity induced by the environmental estrogens. In fact, the transcriptional activity was reduced to a greater extent compared to the AF-1 defective mutant. With bisphenol A, the luciferase activity

was only 27% of the maximal activity by 10^{-9} M estradiol (Fig. 4.2). ß-HCH activated TAF1 at 13% of the maximal estradiol activity while o,p'-DDT was at 54% of the maximal estradiol activity (Fig. 4.4). Octylphenol showed 38% of the maximal estradiol activity (Fig. 4.4). Although the dose-response curves are right-shifted one log, the transcriptional response of TAF1 was saturated by micromolar concentrations of bisphenol A and octylphenol whereas high concentrations of o,p'-DDT and ß-HCH did not saturate the transcriptional activity (Fig. 4.4).

Our experiments show that the transcriptional activity of the AF-2 defective mutant is reduced compared to wild-type receptor. The data suggest that the reduced transcriptional activity using environmental estrogens compared to estradiol is due to different ligand-induced conformations in the AF-2 defective mutant. To increase reporter activity, the TAF1 receptor interacts with the transcriptional machinery through the functioning AF-1 domain. Because the activity is, in general, reduced with environmental estrogens, it implies that the interaction of AF-1 with the transcriptional machinery is reduced. There is evidence that coactivators interact with AF-1 to produce a transactivational response, but the region of AF-1 that mediates this interaction has not been defined to the extent that it has for the AF-2 region (18). It may be that the transcriptional machinery senses the conformation of the estrogen receptor bound by environmental estrogens, thereby producing the tissue selective activity of certain environmental estrogens.

Estrogen receptor with loss of AF-1 and AF-2 function. To measure the activity of the environmental estrogens on an estrogen receptor without either an AF-1 or AF-2 domain, we introduced ER-Null into MDA-MB-231 cells. For every compound and every concentration tested, the luciferase activity was about 2% (Fig. 4.5). MDA-MB-231 cells transfected with the parental pCMV5 plasmid produce a similar amount of luciferase (data not shown). The luciferase activity produced by ER-Null reflects the basal activity of the pS2 minimal promoter. Our data shows that no other cryptic transcription activation functions other than AF-1 and AF-2 are involved in the transcriptional activity of the estrogen receptor in our system (30). Thus, in our co-transfection assay, we are measuring the transcriptional activity of only AF-1 and AF-2 without interference from other transcriptional activation functions present in the receptor.

Estrogen receptor with mutation of serine 118 to alanine: Protein growth factors can produce estrogen-like transcriptional effects (31, 32) and the mechanism may be through MAPK kinase phosphorylation of the estrogen receptor at serine 118 (32, 33). We introduced the S118A mutant receptor into MDA-MB-231 cells since S118A is defective in signaling through the MAPK kinase pathway (33). The phosphorylation of S118 by MAPK kinase produces a ligand-independent increase in transcriptional activity of the estrogen receptor. Since high concentrations of certain environmental estrogens activate phosphorylation pathways, we examined whether the transcriptional activity in wild-type estrogen receptor is due to phosphorylation of S118. We observed that the absolute activity of S118A was similar to wild-type estrogen receptor (data not shown). With estrogen, the luciferase activity increased about five-fold relative to DMSO control and the K_d was approximately $1.0 \bullet 10^{-11}$ M (Fig. 4.6) which is similar for the wild-type receptor since S118A binds estradiol with wild-type affinity.

The S118A transcriptional activity induced by environmental estrogens was similar to activity from wild-type estrogen receptor. With bisphenol A, octylphenol, and ß-HCH the luciferase activity increased four- to five-fold relative to DMSO control (Fig. 4.4). The observed K_d for each of the environmental estogens was similar for the S118A mutant and the wild-type receptor (Figs. 4.1 and 4.6). Similar to wild-type estrogen receptor, 10-5 M o,p'-DDT super activated S118A eight-fold compared to DMSO control (Fig. 4.4). The transcriptional activity was not saturated by high dose o,p'-DDT. Thus, transcriptional activation by estradiol or the xenoestrogens is not dependent upon phosphorylation of serine 118, nor is the super-activation of the receptor by o,p'-DDT due to phosphorylation at serine 118. However, other potential phosphorylation sites are intact in the S118A mutant and other serine mutants must be tested to determine if ligand-independent kinase activation of the estrogen receptor occurs.

Estrogen receptor with a reduction in hormone binding: The mutant R507L binds estradiol with lower affinity for estradiol. We asked whether environmental estrogens utilize the same amino acid residue for ligand dependent binding of compounds. In the experiment, we observed that 10^{-8} M estradiol caused maximal transcriptional activity in R507L (Fig. 4.7). Compared to wild-type estrogen receptor, the dose-response curve of R507L is right-shifted nearly two logs. The R507L receptor had an absolute luciferase activity similar to wild-type estrogen receptor (data not shown). However, since the basal level of transcriptional activity is low, R507L is activated about 60-fold with estradiol (Fig. 4.7) giving R507L a large activation 'window'.

When the environmental estrogens were tested, virtually no activity was seen with bisphenol A, octylphenol, o,p'-DDT, or ß-HCH (Fig. 4.7). Two explanations are possible. First, the amino acid 507 may be critical and required for the binding and transcriptional activity of the environmental estrogens tested. Second, a more likely explanation is that the dose-response curves of the environmental estrogens were right-shifted by two logs. If that is the case, then even at 10^{-5} M none of the compounds are at sufficient concentrations to activate R507L. If larger concentrations of compounds could be achieved perhaps activation would be seen. However, concentrations higher than 10^{-5} M could not be tested since all of the compounds have reached their solubility limits. We conclude that amino acid 507 is required for the activity of weak binding environmental estrogens.

Estrogen receptor with loss of DNA binding activity: To measure the activity of the environmental estrogens on an estrogen receptor without any DNA binding activity, we introduced HE11 into MDA-MB-231 cells. For every compound and every concentration tested, the amount of luciferase activity measured was about 2% which reflects the basal activity of the pS2 minimal promoter (Fig. 4.8). Our data shows that the DNA binding domain is required for transcriptional activity through the ERE2pS2Luc reporter plasmid. It can not be ruled out that environmental estrogens act through other DNA response elements such as an AP-1 site or through a raloxifene response element (34). Further experiments using reporter plasmids containing alternative response elements should answer whether the DNA binding domain is dispensable for transcriptional activation by environmental estrogens.

Estrogen receptor with loss of hormone binding activity: To measure the activity of the environmental estrogens on an estrogen receptor without any hormone binding

activity, we introduced HE48 into MDA-MB-231 cells. The transfected cells were treated with various concentrations of estrogen, bisphenol A, and octylphenol. For every compound and every concentration tested, the amount of luciferase activity measured was about 2% (Fig. 4.9). Our data shows that transcriptional activation by environmental estrogens requires an intact hormone binding domain. Loss of hormone binding results in an absolute loss of transcriptional activation.

5. OTHER: WORK INDIRECTLY SUPPORTED

a. Estrogen Protects against MNU-induced Cataracts

In the first experiment with MNU in ovariectomized rats it was noticed that many of the control animals had developed cataracts by 6 months after MNU treatment but the animals that were treated with estradiol had no cataracts. Therefore the eyes of these animals and those in subsequent investigations were followed. A method was developed for in vitro determination of the degree of opacity of the rat lens and this was used on lenses collected at the end o the tumorigenesis experiment at 8 months. The results of this study were published: Bigsby RM, Cardenas H, Caperell-Grant, A: Estrogen is protective in a rat model of age-related cataracts. Proc Natl Acad Sci, USA 96:9328-32, 1999 (included in the appendix). Briefly the observations indicate that MNU induces the same type of cataract as seen in aging humans and that estradiol and estrone inhibit this effect. Moreover, estradiol was more protective than estrone and the lens was shown to express both types of estrogen receptor, alpha and beta. Since estradiol is the stronger of the estrogen receptor ligands and estrone is the more potent of the chemical antioxidants, it was concluded that the protective effects occurred through an estrogen receptor mediated mechanism and were not due to a non-specific antioxidant property of the steroid.

b. Studies on Estrogen Receptor and Co-activator Expression in the Uterus.

Through the support of the grant Dr. Xinghua Long was able to participate in studies conducted in the lab of Dr. Kenneth Nephew, Indiana University School of Medicine, Bloomington. These studies examined changes in expression of estrogen receptor in response to estrogen treatment and they established the tissue-specific expression of several co-activator proteins involved in estrogen action in the uterus. This work resulted in two publications (listed but not included in the appendix). Although not directly related to breast cancer, these studies reprsent novel determinations of molecular parameters that are likely to regulate estrogen action in target tissue and therefore the results do have relevance to hormonally responsive cancers in general.

c. Studies on the enantiomeric selective accumulation of μ-Hexachlorocyclohexane.

As an offshoot of the studies being performed in the lab of Dr. Hites, the supported graduate student, Elin Ulrich studied the tissue accumulation of different enantiomers of a-HCH. This work was performed by Elin as part of her PhD thesis research and the report was recently published (listed but not included in the appendix). Dr. Ulrich found that the ratio of the (+) enantiomer to (-) enantiomer in the brain was as high as 13.5, indicating a tissue specific accumulation of one enantiomer over the other. In vitro studies suggested that this selective accumulation was not due to a selective metabolism. It is hypothesized that the blood-brain barrier and serum binding proteins are involved in this phenomenon. The importance of this finding is the possible role of enantiomeric specific biological activity of compounds such as a-HCH or o,p'-DDT. The

active ingredient of the pesticide Lindane is g-HCH but this isomer amounts to only 10% of the mixture during manufacture; the a-isomer accounts for 70% of the mixture. The main toxicity of HCH is due to central nervous system damage. From our studies, it is likely that the (+) enantiomer of a-HCH is the causative agent in this toxicity. Others have shown that the (+) enantiomer of o,p'-DDT is more estrogenic than the (-) enantiomer; it would be of interest to determine if there is a selective accumulation of either enantiomer in estrogen target tissues.

d. Studies on strain differences in estrogenic responses to xenoestrogens

During the period of support, Dr. Xinghua Long was also able to participate in a study of the influence of rat strain on expression of estrogenic responses following treatment with the xenoestrogen, bisphenol A. These studies were published (listed but not included in appendix). They showed that BPA caused increased cell proliferation in the vaginal epithelium of Fischer 344 rats but not in Sprague-Dawley rats. This genetic difference in sensitivity to xenoestrogens is important when choosing a model system for studying the potential risks posed by these compounds. It would be wise to choose the more sensitive animal system in order to avoid 'false negative' results.

KEY RESEARCH ACCOMPLISHMENTS:

- For the first time the tumor promoter activity of estradiol and estrone have been defined in the MNU-initiated, ovariectomized adult rat, demonstrating the greater efficacy of estrone.
- A difference in anti-tumorigenesis action of estradiol and estrone in the ovary intact, MNU-initiated rat was demonstrated, with estradiol being the more efficacious.
- A synergistic tumor promoter activity of progesterone plus estrone was demonstrated in the MNU-initiated, ovariectomized rat.
- The lowest blood levels of ß-HCH and o,p'-DDT that produce an observable estrogenic effect were defined in the ovariectomized mouse.
- A requirement for both activation function domains of the estrogen receptor protein to allow full estrogenic efficacy with xenoestrogens was demonstrated.
- A protective effect of estrogen in MNU-induced cataractogenesis was discovered.

REPORTABLE OUTCOMES

Presentations at International Meetings

Bigsby RM: Molecular and Pharmacokinetic Mechanisms of Xenoestrogen Action, Keystone Symposium on Endocrine Disruptors, Tahoe City, CA, February, 1999

Long X, Nephew KP, Young PCM, Bigsby RM Strain differences in sensitivity of rat vagina to estrogenic effects of bisphenol A. Keystone Symposium on Endocrine Disruptors, Tahoe City, CA, Feb. 1999.

Gize EA, Yang NN, Bigsby RM Environmental estrogens activate AF-1 and AF-2 estrogen receptor-alpha mutants uniquely compared to estradiol. Keystone Symposium on Endocrine Disruptors, Tahoe City, CA, Feb. 1999.

Bigsby RM, Caperell-Grant A, Cardenas H, Protective effects of estrogen in a rat model of age-related cataracts. US-Japan Combined Cataract Research Group Meeting, Kona, HI, November, 1999

Bigsby RM: Molecular and Pharmacokinetic Mechanisms of Xenoestrogen Action, International Symposium on Environmental Endocrine Disruptors, Kobe, Japan, December 1999.

Published Abstracts

Bigsby RM, Caperell-Grant A Estrogen protects against cataractogenesis in NMU-treated rats. 81st Annual Meeting of the Endocrine Society, San Diego, CA, June, 1999.

Nephew KP, Long X, Osborne E, Burke KA, Ahluwalia A, Bigsby RM Cell type-speicific down regulation of the estrogen receptor by estradiol in rat uterus. 81st Annual Meeting of the Endocrine Society, San Diego, CA, June, 1999.

Ulrich EM, Caperell-Grant A, Hites RA, Bigsby RM: Correlation between xenoestrogen tissue concentration and biological response in mice. Annual Meeting, Society of Environmental Toxicology and Chemistry, Philadelphia, PA, November 1999.

Ulrich EM, Caperell-Grant A, Hites RA, Bigsby RM: Xenoestrogen tissue concentrations correlated biological responses in mice. Dioxin '99, Venice, Italy, September 1999.

Publications/Manuscripts

Ulrich EM, Caperell-Grant A, Hites RA, Bigsby RM: Environmentally relevant xenoestorgen tissue concentrations correlated to biological responses in mice. Environ Health Perspect 108:973-7, 2000

Bigsby RM: Synergistic tumor promoter effects of estrogens and progesterone in methylnitrosourea-induced rat mammary cancer. Carcinogenesis, submitted

Gize EA, Bigsby RM Both activation domains of the estrogen receptor are required for full xenoestrogen action, in preparation.

Other Publications - not directly related to Statement of Work ,but indirectly supported by award.

Bigsby RM, Cardenas H, Caperell-Grant, A: Estrogen is protective in a rat model of age-related cataracts. Proc Natl Acad Sci, USA 96:9328-32, 1999

Nephew KP, Long, X, Osborne EZ, Burke KA, Bigsby RM: Effect of estradiol on estrogen receptor expression in rat uterine cell types. Biol Reprod 62:168-77, 2000.

Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PCM, Nephew KP, Bigsby RM: Strain Differences in Vaginal Responses to the Xenoestrogen, Bisphenol A, Environ Health Perspect, Environ Health Perspect. 108(3):243-247, 2000

Nephew KP, Ray S, Hlaing M, Ahluwalia A, Wu SD, Long X, Hyder SM, Bigsby RM: Expression of estrogen receptor coactivators in the rat uterus Biol. Reprod 63:361-367, 2000.

Ulrich EM, Willett KL, Caperell-Grant A, Bigsby RM, Hites RA: Understanding enantioselective processes: A laboratory rat modelfor a-hexachlorocyclohexane accumulation. Environ sci Technol 35:1604-9, 2001

III. CONCLUSIONS

Although the standard MNU-initiated, adult rat model would seem to be the ideal model system for doing so, the tumor promoter effects of ovarian hormones has never been established in this system. Our studies have shown that stimulation of mammary gland growth by estradiol or estrone is not in itself sufficient to promote tumorigenesis to same extent as the intact ovary. In addition, it appears that there is a biphasic response to estrogens: they are able to both promote and inhibit tumorigenesis. Although it was considered controversial (35), it may be that progesterone supplied by the ovary is also essential for full tumor promoter activity. This was tested in the ovariectomized rat. Progesterone alone had no promoter effect but it synergistically enhanced estrone's tumor promoter activity. This supports the recent epidemiological findings that combined hormone replacement therapy, estrogen plus progestin, is associated with an increased risk for breast cancer (36, 37) and it indicates that the MNU-initiated , ovariectomized rat is an important model in which to continue the study of this phenomenon.

The serendipitous finding that estrogens, particularly estradiol, can protect against cataracts is important on a number of levels. First, it supports the epidemiological data suggesting that prolonged life-time exposure to estrogen reduces risk for age-related cataracts. Second, it shows that this model system can be used for further study of this phenomenon.

The studies on estrogenic effects of op-DDT and ß-HCH in the mouse reproductive tract have produced some startling results. This is the first study in which blood levels of test compound were correlated to estrogenic response. In past reports xenoestrogens were administered in very large doses (100-500 mg/kg BW) and acute estrogen responses were monitored (4, 38-41). The impression from such studies was that xenoestrogens, being very weakly estrogenic by a number of *in vitro* assays (41-44), required the very large doses of compound to have an effect *in vivo*. In our study the compounds were delivered continuously in low doses. The blood levels attained by the highest doses in the mice were well above those which are found in the general population of people. However, the lowest dose that induced significant biological effects in the mice produced blood levels that were within the same order of magnitude of blood levels found in some 'non-exposed' humans (45). Thus, under the condition of a continuous low level of exposure, estrogenic effects can be expected from levels of xenoestrogen commonly encountered in humans.

The MCF-7 xenograft studies have been inconsistent and no conclusions can be drawn at this date. The tumors formed from the cells now available do not seem to have the same growth characteristics as those of our earlier study. And xenoestrogens have inconsistently stimulated tumor growth. In the single experiment in which food restriction was applied, the xenoestrogen was ineffective with or without dietary restriction.

The studies on the estrogen receptor domains which participate in estrogen and xenoestrogen transcriptional activation have given us some clues as to how compounds can produce qualitatively and quantitatively disparate effects in the same tissue. Compounds such as BPA, octylphenol, and ß-HCH are fully effective compared to

estradiol when the wild-type receptor is present; o,p'-DDT produces a super-activation of the reporter gene. Deletion of either AF-1 or AF-2 results in a loss of relative efficacy of the xenoestrogens. Thus, they appear to require a synergy between the two activation function domains. Recent observations by Nishikawa (46) suggest that the different compounds are likely to produce different receptor–coactivator interactions. The fact that o,p'-DDT consistently produced a greater response than estradiol suggests that it may enhance binding of coactivator to receptor. Such differences in molecular activity of compounds should be considered when attempting to assess their potential bioactivity.

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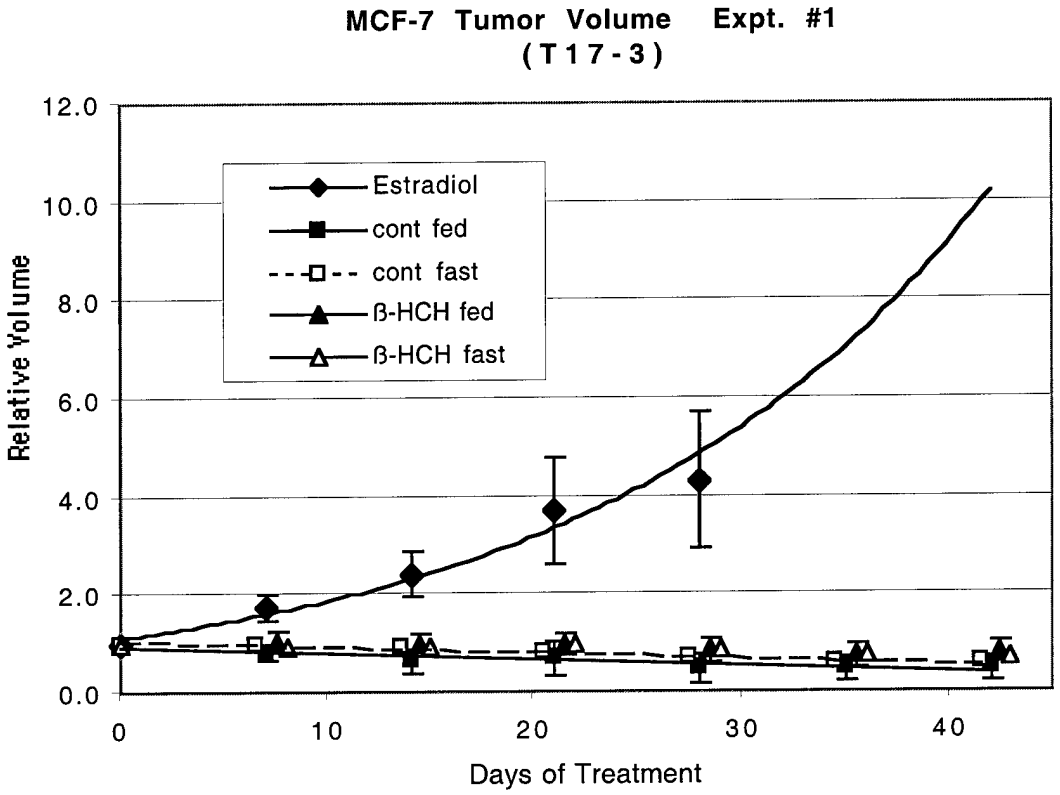

Figure 2.1. Effect of ß-HCH on MCF-7 Tumor Growth in Athymic Mice on Dietary Restriction. MCF-& tujor was transplanted to mice that were under estrogen stimulation via a subcutaneous capsule of estradiol. After 3 weeks the estradiol capsule was replaced with an empty control capsule (cont) or one containing ß-HCH (ß-HCH). Half of these hosts were placed on periodic dietary restriciton (fast) while the others were left on food ad libitum (fed).

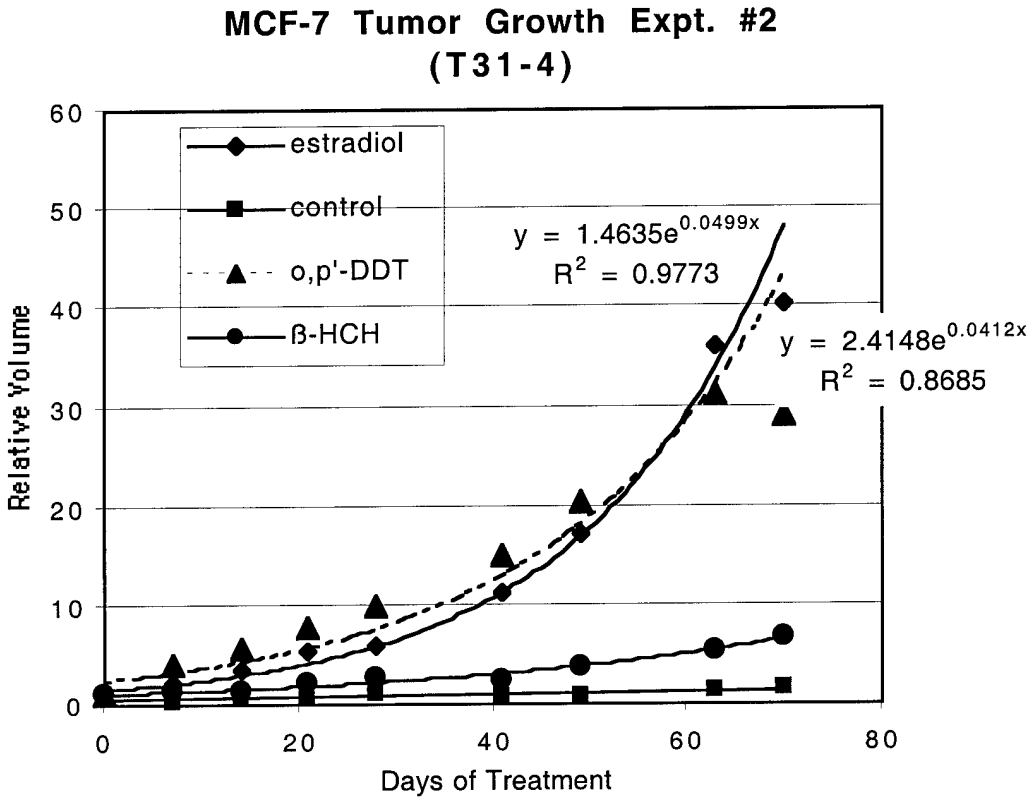

Figure 2.2. Effect of o,p'-DDT or ß-HCH on MCF-7 Tumor Growth. Tumor was transplanted from a host that had not been treated with estrogen to maintain the tumor's growth. This tumor was grown for 5 weeks in ovary intact animals with only 3 daily injections of estradiol administered at the time of transplantation.

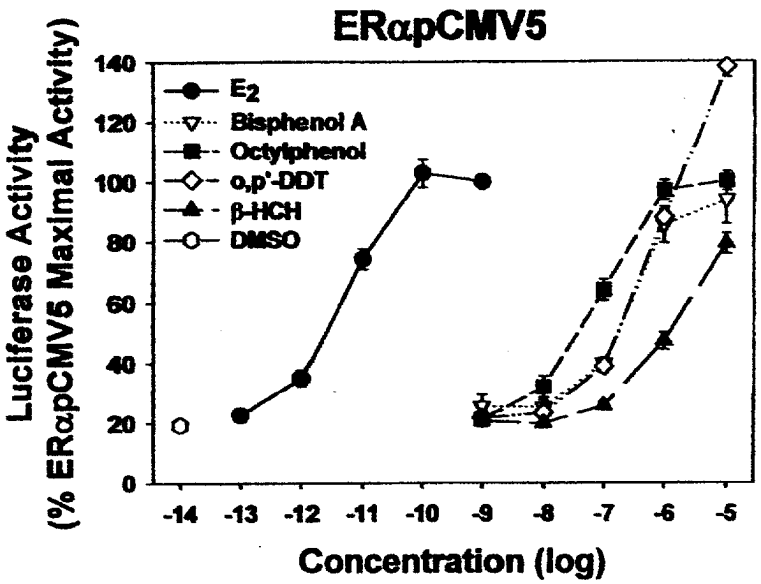

Fig. 4.1 Transfection of MDA-MB-231 cells with wild-type ERα. ERα negative MDA-MB-231 cells were co-transfected with the ERαpCMV5 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH, and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to the ERαpCMV5 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

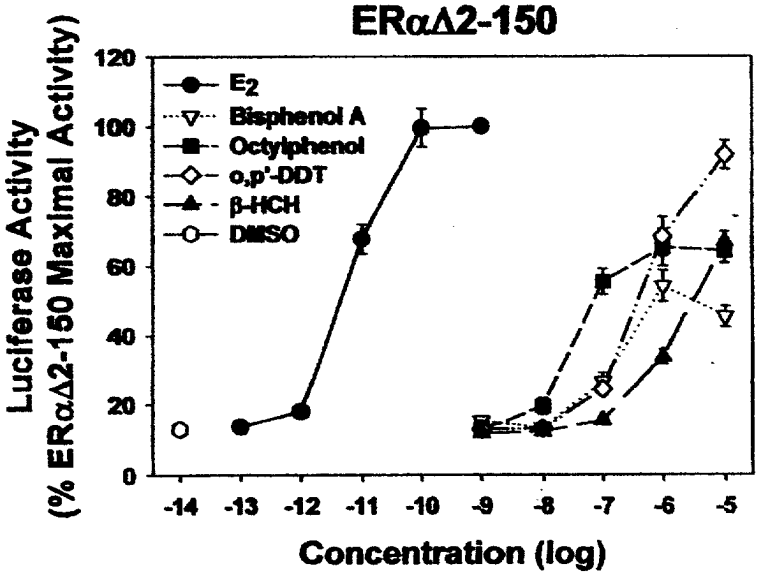

Fig 4.2 Transfection of MDA-MB-231 cells with an ERα mutant with loss of AF-1 function. ERα negative MDA-MB-231 cells were co-transfected with the ERαΔ2-150 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to ERαΔ2-150 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

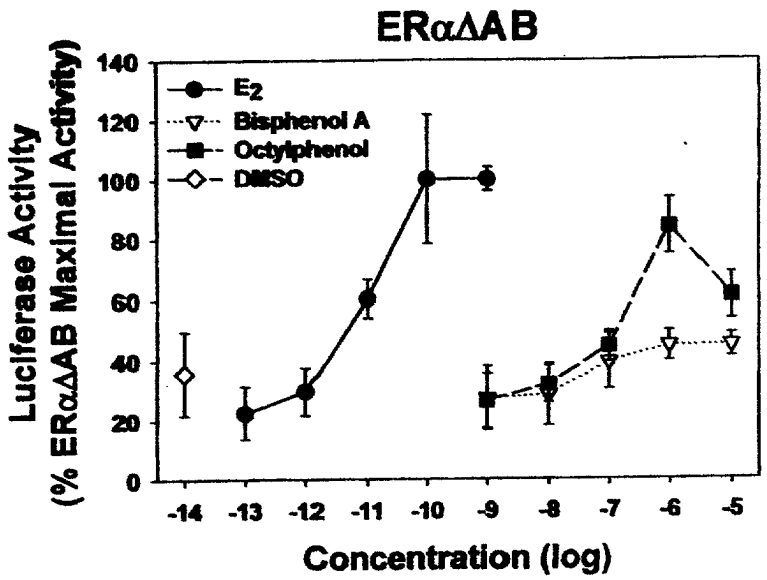

Fig 4.3 Transfection of MDA-MB-231 cells with an ERα mutant with loss of AF-1 function. ERα negative MDA-MB-231 cells were co-transfected with the ERαΔAB plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to ERαΔAB 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

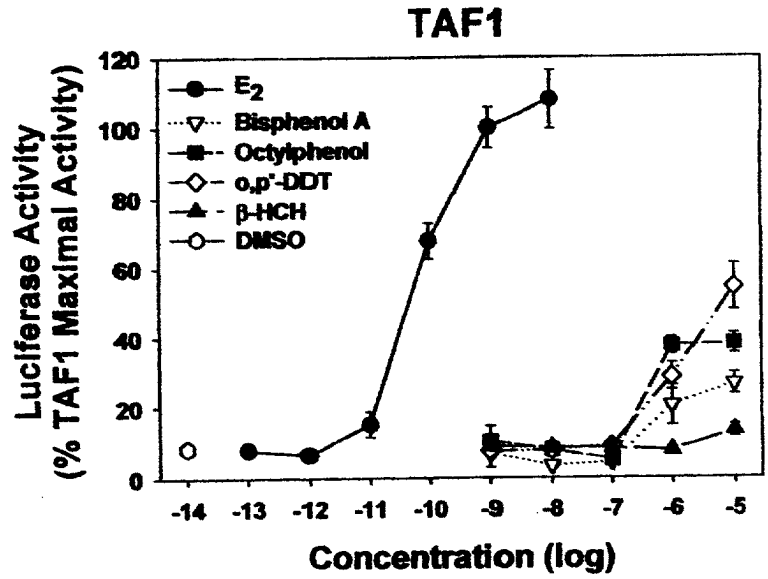

Fig 4.4 Transfection of MDA-MB-231 cells with an ERα mutant with loss of AF-2 function. ERα negative MDA-MB-231 cells were co-transfected with the TAF1 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to TAF1 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

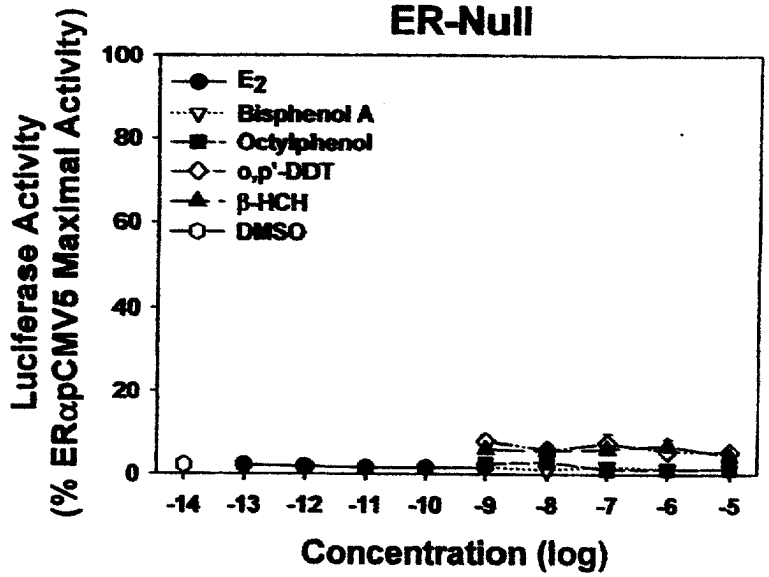

Fig. 4.5 Transfection of MDA- MB-231 cells with an ERα mutant with loss of AF-1 and AF-2 functions. ERα negative MDA-MB-231 cells were co-transfected with the ER-Null plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to ERαpCMV5 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

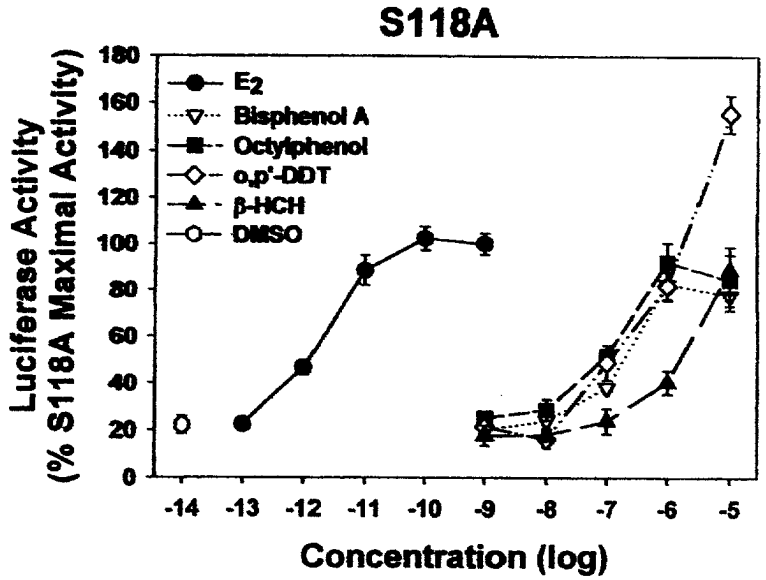

Fig. 4.6 Transfection of MDA-MB-231 cells with S118A. ERα negative MDA-MB-231 cells were co-transfected with the S118A plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to S118A 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

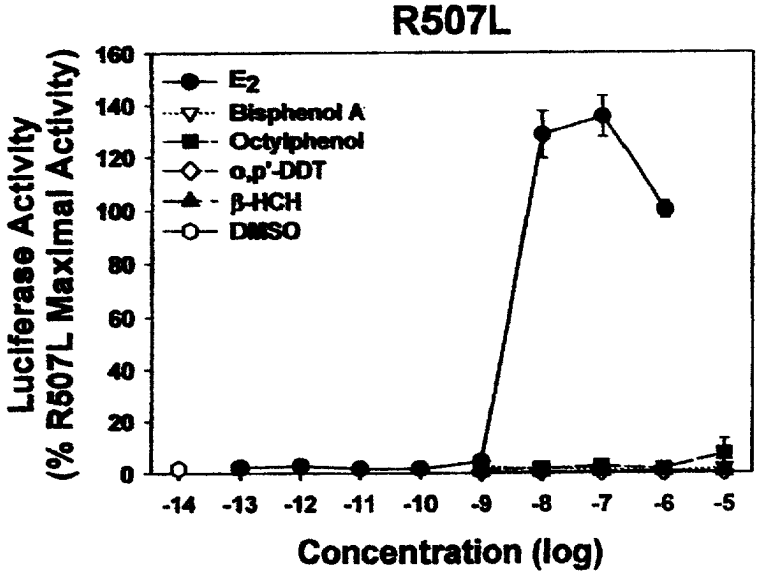

Fig. 4.7 Transfection of MDA-MB-231 cells with an ERα mutant with a reduction in binding affinity for estradiol. ERα negative MDA-MB-231 cells were co-transfected with the R507L plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to R507L 10^{-6} M E_2 maximal response being 100% from three separate experiments in triplicate.

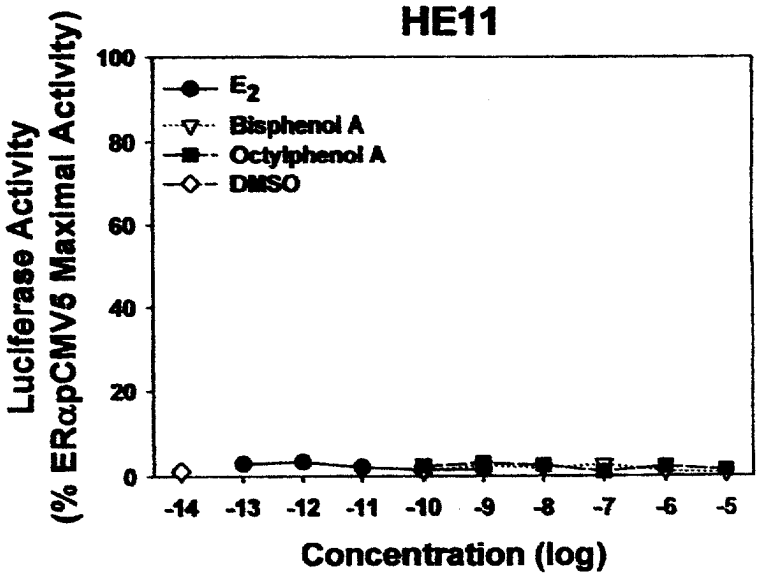

Fig. 4.8 Transfection of MDA-MB-231 cells with an ERα mutant with loss of DNA binding activity. ERα negative MDA-MB-231 cells were co-transfected with the HE11 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to ERαpCMV5 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

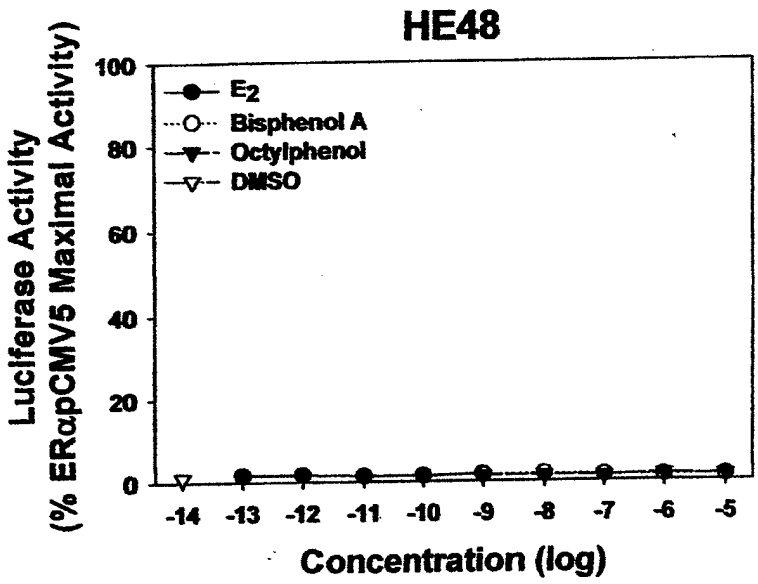

Fig.4.9 Transfection of MDA-MB-231 cells with ERα mutant with loss of hormone binding activity. ERα negative MDA-MB-231 cells were co-transfected with the HE48 plasmid, the ERE2pS2Luc reporter plasmid, and the pAD plasmid as internal control. Cells were treated with various concentrations of E_2, Bisphenol A, Octylphenol, o,p'-DDT, β-HCH and DMSO for 24 h. Luciferase activity was normalized to β-galactosidase activity. The values represent the mean ± SEM relative to ERαpCMV5 10^{-9} M E_2 maximal response being 100% from three separate experiments in triplicate.

APPENDIX

REPRINTS:

Bigsby RM, Cardenas H, Caperell-Grant, A: Estrogen is protective in a rat model of age-related cataracts. Proc Natl Acad Sci, USA 96:9328-32, 1999 [3 double sided pages]

Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PCM, Nephew KP, Bigsby RM: Strain Differences in Vaginal Responses to the Xenoestrogen, Bisphenol A, Environ Health Perspect, Environ Health Perspect. 108(3):243-247, 2000 [3 double sided pages]

Ulrich EM, Caperell-Grant A, Hites RA, Bigsby RM: Environmentally relevant xenoestorgen tissue concentrations correlated to biological responses in mice. Environ Health Perspect 108:973-7, 2000 [3 double sided pages]

Ulrich EM, Willett KL, Caperell-Grant A, Bigsby RM, Hites RA: Understanding enantioselective processes: A laboratory rat modelfor a-hexachlorocyclohexane accumulation. Environ sci Technol 35:1604-9, 2001 [3 double sided pages]

SUBMITTED MANUSCRIPT:

Bigsby RM: Synergistic tumor promoter effects of estrogens and progesterone in methylnitrosourea-induced rat mammary cancer. Carcinogenesis, submitted [25 pages]

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Protective effects of estrogen in a rat model of age-related cataracts

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ABSTRACT **Women have a higher incidence of cataracts, and epidemiologic data suggest that the increased risk may be caused by a lack of estrogen in postmenopausal years. We have examined the effects of estrogen on methylnitrosourea (MNU)-induced cataractogenesis in Sprague–Dawley rats. Animals were ovariectomized, injected with MNU, and treated with estradiol or estrone by a continuous-release, subcutaneous Silastic implant, or they received an empty Silastic implant (no hormone). In the no-hormone group, rats developed opaque lenses approximately 6 months after MNU treatment. By 8 months, 74% (14/19) of the no-hormone rats had evident opacity in one or both eyes by simple gross inspection; 58% (22/38) of the eyes in this group were opaque. Estradiol or estrone treatment reduced the incidence of cataractous eyes to 12% or 25%, respectively. Lenses were examined under a dissecting microscope for light transmission. The lenses of the group treated with no hormone had light transmission of 26% ± 9.2%, whereas lenses from the estradiol-treated animals had light transmission of 72% ± 5.8%. Histological examination revealed that the anterior cortices of the opaque lenses were disrupted and showed the hallmark signs of age-related cataracts; in addition, some eyes that appeared clear by macroscopic examination showed the early histologic signs of cataractogenesis. It was demonstrated with reverse transcription–PCR that lens cells express both α and β types of estrogen receptor, suggesting that the protective effects of the hormones may be a direct, receptor-mediated phenomenon. Thus, the MNU-treated, ovariectomized rat serves as a model for age-related cataractogenesis, and observation of a clear protective effect of estrogens in this system supports the implications of epidemiologic data.**

More than 75% of people ≥75 years old have some degree of lens opacification (1), and it is estimated that >50% of blindness is caused by cataracts (2). Age-related cataracts can be classified according to their anatomic location within the lens: cortical, nuclear, posterior, or mixed (3). Women exhibit an increased incidence of cataracts compared with age-matched men (4–7), mainly because of a higher rate of cortical cataracts (8, 9). Thus, age-related cataracts present a significant health problem, one that exhibits a sexual dichotomy.

Epidemiologic evidence suggests that estrogens may protect against cataracts. Although women are at a higher risk of developing cataracts than are men, this increased risk comes after menopause, when estrogen levels have waned (9, 10). In one study of 544 women, early onset of menopause was associated with a 2.9-fold risk of developing cataracts (11). Moreover, the results of three small epidemiologic studies suggest that postmenopausal estrogen replacement therapy reduces the incidence of cataracts (12–14). The role of estrogen in modifying the onset of age-related cataractogenesis requires suitable experimental models for further study.

Results of animal studies have produced conflicting observations, but overall, they suggest that a lack of estrogen is associated with cataractogenesis. Rats treated with oral contraceptives containing estrogen and progestogen have an increased incidence of cataracts compared with nontreated controls, suggesting that estrogens may actually promote cataract formation (15). However, the effectiveness of estrogen in these treatments is not certain, because the uteri of the treated animals were atrophic (15); progestogen may have produced a predominantly antagonist effect. In another study, long-term treatment with the antiestrogen tamoxifen increased the incidence of cataracts in rats (16). However, tamoxifen exerts both antagonist and agonist estrogen activity in different end organs (17), making conclusions about the mechanism of its cataract-promoting action difficult to draw. A brief report of cataractogenesis in transgenic mice expressing a dominant negative form of the estrogen receptor (ER) supports the notion that inhibition of estrogen action promotes cataractogenesis (18), but the negative dominance of the mutant ER required that it be activated by endogenous estrogen supplied by the ovaries or by treatment of ovariectomized animals with the potent synthetic estrogen diethylstilbestrol. Thus, although these studies point to an association of decreased estrogen action with increased cataractogenesis, they contain caveats that make definitive conclusions difficult.

We report here on a clear protective effect of estrogen in a rat model of accelerated, age-related cataractogenesis. The tumor initiator methylnitrosourea (MNU) causes cataracts to appear 6 to 8 months after a single intravenous injection into outbred rats (19). MNU induces cortical cataracts with many of the hallmarks associated with age-related cataracts in women. We show that a much reduced incidence of cataractogenesis occurs when estrogen is supplied to ovariectomized rats that have been treated with MNU.

METHODS

Animal Treatments. All procedures performed on experimental animals were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine. Sixty female, 49-day-old Sprague–Dawley rats (Harlan, Indianapolis, IN) were received. Within one week, 50 animals were ovariectomized (one group of 10 rats was not ovariectomized) and all were treated while under general anesthesia induced with ketamine. Each animal received a single intravenous injection of 50 mg/kg MNU (Sigma), and a treatment Silastic capsule was placed subcutaneously on the back. MNU was dissolved in PBS and injected intravenously

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Abbreviations: MNU, methylnitrosourea; ER, estrogen receptor; RT-PCR, reverse transcription–PCR.
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through the tail within 15 min of preparing the solution. Silastic capsules containing approximately 20 mg of crystalline estradiol (E2) (20) were applied to 20 ovariectomized animals; likewise capsules containing 20 mg of estrone were implanted into 10 ovariectomized animals. Twenty ovariectomized rats received an empty Silastic capsule; these are referred to as the "no-hormone" treatment group. The 10 ovary-intact animals were anesthetized and injected with MNU. Animals were observed on a weekly basis by simple visual inspection for any gross changes in eye appearance. Animals were carried through 40 weeks after MNU treatment before being killed. Serum estradiol levels were determined with a solid-phase radioimmunoassay as described earlier (21) in 5 animals taken at random from each of the hormone and the no-hormone treatment groups.

Lens Histology. Entire eyes were removed from animals, slit open across the cornea, and immersed in fixative (neutral formalin/ethanol/acetic acid/water, 2:3:1:3) for 2 weeks, as described by Roy *et al.* (19). These eyes were processed and embedded in paraffin. Six-micrometer sections were prepared and stained with hematoxylin and eosin.

Light Transmission Through Lenses. The eyes of seven estradiol-treated, ovariectomized animals and seven no-hormone, ovariectomized animals were extruded and slit open around the cornea, and the lenses were carefully removed. The lens from each eye was placed in a shallow culture dish containing PBS. The dish was placed on the stage of a dissecting microscope with its zoom objective lens set at 1.5×; a charge-coupled device color video camera (Model DXC-960MD, Sony) was attached to one ocular. The lens was viewed with transmitted light and the image was captured by using an imaging program (IPLAB SPECTRUM, Signal Analytics, Vienna, VA) run on a computer (Macintosh Power PC, Apple). A 2-mm-thick piece (1 cm square) of opaque, white Teflon was included in the microscopic field for measurement of zero transmitted light. The intensity of the light (in arbitrary units) transmitted at the center of the lens was measured with the IPLAB SPECTRUM program. Likewise, the intensity of light transmitted through the culture dish to a position just outside the lens was measured and used to define 100% transmission. The units of light intensity measured from the Teflon piece were considered background and used to correct the light transmission measurements made at the lens and outside the lens. The light passing through the lens was calculated as the percentage transmission.

ER Reverse Transcription (RT)-PCR. Lenses were collected from six adult rats. The lenses were immediately frozen in liquid nitrogen, pulverized, and homogenized to extract total RNA by using a kit (RNeasy, Qiagen, Chatsworth, CA). A sample of lens RNA (0.5 μg) was subjected to RT, primed by random hexamer oligonucleotides, and subjected to PCR (Gene Amp RNA PCR, Perkin–Elmer). Aliquots of the RT reaction were used in PCRs for ERα with oligonucleotide primers kgb5 and kgb6, and for ERβ with oligonucleotide primers erbkg1 and erbkg2, as described by Kuiper *et al.* (22); these primer pairs yield amplicons of 344 bp and 262 bp for ERα and ERβ, respectively. As positive controls, 0.5 μg of total RNA from uterus and prostate were subjected to the same RT–PCR procedures. As a negative control, lens RNA that was not subjected to the RT reaction was used in PCR with both primer sets. PCR was carried out over 35 cycles of 95°C for 1 min, 55°C for 45 sec, and 72° for 2 min, followed by 7 min at 72°C for product extension. The products of the RT–PCRs were subjected to electrophoresis through 1.0% agarose and visualized by ethidium bromide fluorescence. The ERα and ERβ amplicons from lenses were ligated into a bacterial plasmid with the TA Cloning System (Invitrogen), and the cloned cDNAs were sequenced and analyzed at the Indiana University School of Medicine Biotechnology Facility to verify their origin.

Statistics. The incidence of gross lens opacities, as identified by visual inspection, was compared by χ^2 analysis (23). The means of light transmission through lenses were compared by *t* test (STATVIEW, Abacus Concepts, Berkeley, CA).

RESULTS

One of the no-hormone, ovariectomized animals died before the end of the experiment and three of the estradiol-treated animals were killed early because of the presence of large tumors: two animals had mammary tumors and one had a salivary gland tumor. At the end of the 8-month experimental period, treatment capsules still contained approximately one-half of the original mass of crystalline steroid. Serum estradiol levels were 23.0 ± 1.24 pg/ml or 12.5 ± 2.53 pg/ml (±SEM) for estradiol-treated or estrone-treated animals, respectively; the no-hormone, ovariectomized animals had serum estradiol levels of 0.72 ± 0.42 pg/ml.

Gross examination first revealed cataracts in the ovariectomized animals approximately 6 months after MNU injection (Fig. 1). Treatment of ovariectomized animals with either estradiol or estrone significantly reduced the incidence of cataracts (Table 1). By 8 months, 74% (14/19) of the no-hormone, ovariectomized animals had cataracts; of the 14 animals with cataracts, 8 had bilateral cataracts. The estradiol-treated group had a significantly lower incidence ($P < 0.01$)

FIG. 1. Gross examination of rat eyes. (*Right*) An ovariectomized animal with one opaque eye. (*Left*) An animal that had been treated with estradiol is devoid of cataracts.

Table 1. Effects of estrogen on the incidence of lens opacities in ovariectomized, MNU-treated rats

Treatment	Incidence of cataracts			
	Animals	%	Eyes	%
No hormone	14/19	74	22/38	58
Estradiol	3/17	18**	4/34	12**
Estrone	4/10	40	5/20	25*

Within a treatment group, the proportion of animals (%) with either one or both eyes opaque, and the proportion of eyes (%) that were opaque are shown. The values for the estradiol-treated group and the estrone-treated group were compared separately against the no-hormone group by χ^2 analysis: *, $P < 0.05$; **, $P < 0.01$.

with 18% (3/17) of animals having obvious cataracts by visual inspection; only 1 animal had bilateral cataracts. Although the percentage of eyes with cataracts decreased with estrone treatment (25% for estrone-treated vs. 58% for control, $P < 0.05$, Table 1), the number of animals with cataracts in the estrone-treated group (4/10) was not statistically different from the controls. Most of the ovary-intact, MNU-treated animals had large mammary tumors and were killed before 5 months after treatment; one of the two intact animals that survived beyond 5 months had cataracts at the time of death.

The degree of lens opacity varied from eye to eye. The amount of light that was transmitted through isolated lenses was measured in eyes from seven control animals and seven estradiol-treated animals chosen at random at the time of death. The range in the level of clarity among lenses is depicted in Fig. 2 *A–E*. When a clear lens is placed over a grid and transilluminated, the underlying grid lines are obvious in the image (Fig. 2*A*); light transmission for such lenses was measured at 96% to 100%. Loss of clarity and light transmission varied widely, as shown in Figs. 2 *B–E*; some lenses, e.g., that seen in Fig. 2*E*, had 0% light transmission. The average light transmission differed between the control and estradiol-treated groups ($P < 0.01$). In the no-hormone group, the 14 lenses examined had percent transmissions over a range of 0% to 91% with an average of 26% ± 9.2% (±SEM). Lenses from the estradiol-treated animals exhibited an average of 72% ± 5.8% transmission (Fig. 2*F*); this latter group included one lens with a 12% light transmission whereas the remainder transmitted light at 51% to 92%.

Histological examination of clear and opaque lenses revealed that the opacities were due mainly to disruption of the cortex. The anterior cortex of the opaque lenses showed various degrees of disruption, with the most severe cases exhibiting balloon cells and liquefaction of the tissue (Fig. 3*B*). A lens taken from an estradiol-treated animal appeared clear on gross examination, but the nucleated fibers in the bow area of the equatorial region showed swelling and had a granular appearance, which are early signs of cataractogenesis (Fig. 3*C*). In the cataractous lens, the epithelium in the equatorial region was hyperplastic, producing a multilayered tissue (Fig. 3*D*).

Expression of ER within lens cells was assessed by RT–PCR analysis. As shown in Fig. 4, mRNAs for both ERα and ERβ were present; slightly more PCR product appears for ERα. No bands were present when the reverse transcriptase step was omitted (not shown), indicating that no genomic DNA contamination of the RNA occurred that would produce a false-positive result. Sequence analysis of the cloned amplicons verified that they represented ERα and ERβ. Similar analysis of the same amounts of RNA from rat prostate and uterus was used to validate the RT–PCR procedure. It is known that the prostate expresses high levels of ERβ and lower levels of ERα, whereas the uterus expresses high levels of ERα and little or no ERβ (22, 24). Our RT–PCR analysis reflected these relative expression levels in the prostate and uterus (Fig. 4).

DISCUSSION

In this report, an animal model of age-related cataractogenesis showed estrogen replacement to be protective. The induction of cataracts in rats by MNU was described in an earlier report but the sex of the animals used in those studies was not indicated (19); however, it is unlikely that females were used because MNU induces hormone-dependent mammary tumors in rats (25). The rate of growth of such tumors in ovary-intact animals would preclude carrying the animals through the 8 months of observation required (ref. 25 and this study). Our histological results indicate that the cataracts that develop in the MNU-treated rat are similar to those that develop in aging animals (26) and in postmenopausal women (27). In both cases, anterior cortical opacification occurs with the histological hallmarks of cellular swelling, balloon cells, vacuolization, and liquefaction. We also found hyperplasia of the equatorial epithelial cells, which has been described in posterior capsular cataracts in humans (28). Thus, the MNU-treated ovariectomized rat appears to be a suitable model of cataractogenesis

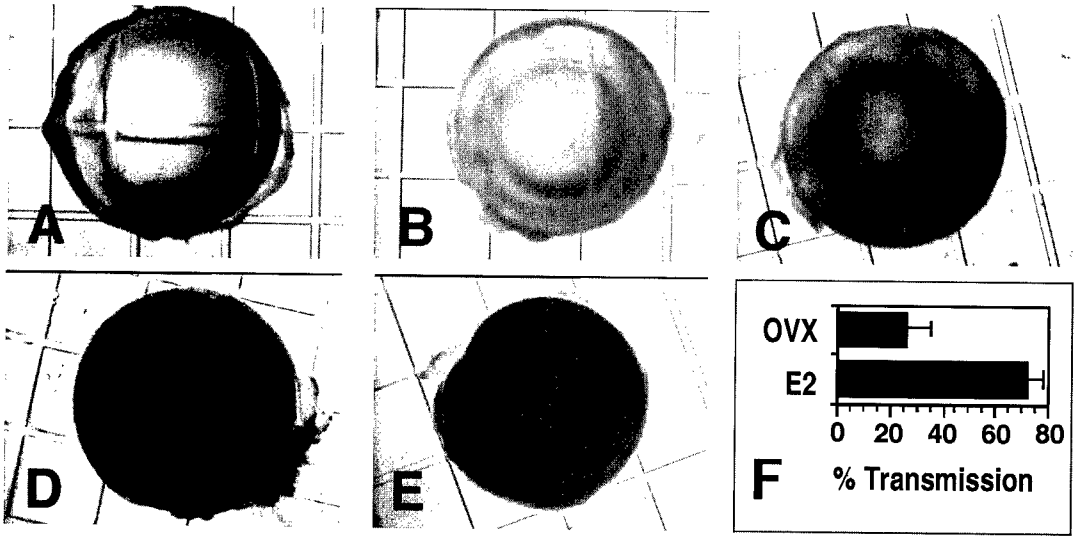

FIG. 2. Transillumination of isolated lenses. Lenses were placed in a culture dish that had a grid etched in its bottom surface. The dish was placed on the stage of a dissecting microscope and viewed with transmitted light. Eyes that appeared clear (*A*) or that had various degrees of opacity (*B–E*) by gross examination showed a gradation of clarity and light transmission. (*F*) The average light transmission of lenses from the no-hormone (OVX) and the estradiol-treated (E2) animals; the means differed when compared by *t* test ($n = 14$; $P < 0.01$; error bars = SEM).

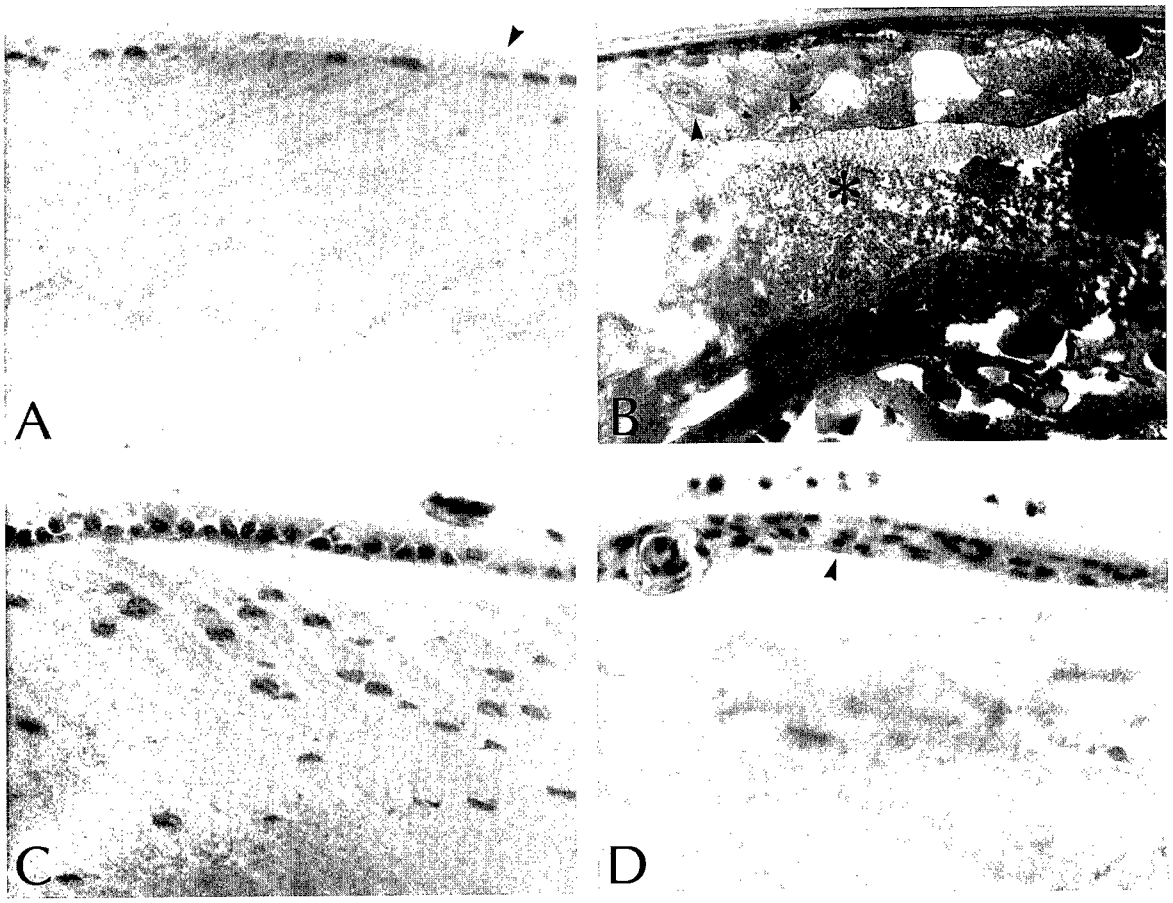

FIG. 3. Histology of lenses from clear or cataractous eyes. (*A* and *C*) Lenses from an estradiol-treated animal. (*B* and *D*) Lenses from a no-hormone animal. The eye of the estradiol-treated animal appears clear on gross examination, whereas the eye of the no-hormone-treated animal is opaque. (*A*) The anterior cortex of the estradiol-treated animal has a homogenous appearance and is covered by a lens capsule made of a thin epithelial cell layer and a normal, thick lens capsule (arrowhead). (*B*) The anterior cortex of the opaque eye is disrupted with the appearance of balloon cells (arrowhead) and areas of complete fiber degeneration and liquefaction (asterisk); the lens capsule is normal in appearance. (*C* and *D*) In the equatorial region, the clear lens (*C*) exhibits swelling of the nucleated fibers in the bow area and the fibers have a slight granular appearance. In the opaque lens (*D*), the fibers in the bow area are disrupted and the lens epithelium is hyperplastic (arrowhead). [Magnification = 250× (*A, C,* and *D*) and 125× (*B*).]

in postmenopausal women and should be useful in determining the mechanisms of the protective effects of estrogen.

As in humans, the incidence of cataracts in rats increases progressively with age, with the majority of lesions occurring after 14 months (26, 29). At 2 years of age, 11% of Sprague–Dawley rats exhibited some form of cataract, but the incidence of anterior cortical lesions was only 0.8% and 2.0% at 14 and 24 months, respectively (29, 30). MNU may enhance the normal aging processes that lead to lens opacification and

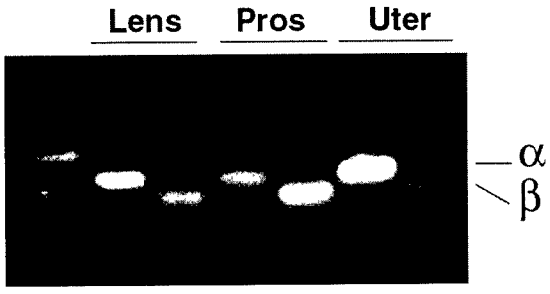

FIG. 4. Demonstration of ERα and ERβ expression in the lens by RT–PCR. Samples of total RNA (0.5 μg) extracted from rat lenses (Lens), prostate (Pros), or uterus (Uter) were subjected to RT–PCR for ERα (α) and ERβ (β). The reaction products were electrophoresed through a 1.0% agarose gel and stained with ethidium bromide. The relative intensities of α and β amplicons in the uterus and prostate reflect the known patterns of expression in these organs.

estrogen may have slowed this process. In the present study, the estrogen-treated groups were not completely devoid of cataracts. Microscopic examinations indicated that even the lenses that appeared clear macroscopically did not always fully transmit light, and some showed early histological signs of cataractogenesis. It will be important to carry these studies forward to determine whether the estrogen-treated animals show an increasing incidence of cataracts as they age beyond the 8 months after MNU treatment.

The mechanisms by which MNU induces cataracts are unknown. MNU is rapidly oxidized in a neutral solution and must be applied within minutes of preparation to maintain its carcinogenic effectiveness; it is fully degraded and cleared from the blood within just 15 min (31). MNU is an alkylating agent that produces DNA adducts in tissues within minutes of its administration to rats (32); although some forms of this DNA damage are repaired (33, 34), the DNA adducts may prove to be very persistent in specific tissues (35). MNU is also capable of acting as a methyl-group donor for glutathione and cysteine (36) and thus may produce protein adducts, although such findings have not been described. It has been suggested that alkylating agents are cataractogenic because of interference with cell proliferation (37) or gene expression (38). A determination of the type and persistence of adducts formed in the lens will be important to understanding how MNU induces cataracts.

We can only speculate about the mechanisms involved in the protective effects of estrogen. We have demonstrated that rat

lens cells express both ERα and ERβ, but it is not known whether presence of the receptor is required for the protective effects of the hormone. Although reports have linked use of the antiestrogen tamoxifen with an increased incidence of cataracts (39), a lack of any such association was also reported (40). Experimental studies have shown that tamoxifen (16) and the related antiestrogen clomiphene (41) induce cataracts in rats. Zhang and coworkers (42) have shown that tamoxifen can induce lens opacification during short-term incubation in explant culture of lenses. These observations suggest that the ER does play a role in protection against cataractogenesis. However, the *in vitro* studies of Zhang *et al.* (43) suggest that the cataractogenic effect of tamoxifen is not mediated by the ER, but rather involves its ability to block chloride channels, thereby causing excess hydration of lens fibers (44).

On the other hand, Katzenellenbogen and coworkers have found that the ER enhances transcription of the gene for a Na^+/H^+ exchanger regulatory factor (45); in this way, estrogen may aid in the maintenance of proper ionic composition and cell hydration through a genomic effect. And finally, it is well established that antioxidants are protective in experimental cataractogenesis (for review, see ref. 46). Estrogens can behave as antioxidants either through a purely chemical, nongenomic mechanism (47) or through enhanced transcription of genes such as quinone reductase (48). However, the antioxidant hypothesis of the protective effect of estrogen is incongruent with the observation that antiestrogens, which are also chemical antioxidants (49), induce cataracts (16, 41). In addition, chemical antioxidant effects are likely to require high concentrations of steroid, but the serum levels achieved by the hormone treatments were in a physiologic range. Further study is required to determine whether genomic or nongenomic mechanisms mediate the protective effects of estrogen in cataractogenesis.

In summary, we have shown that estrogens are protective in an animal model of cataractogenesis. These studies provide strong experimental evidence supporting the suggestion, derived from recent epidemiologic studies (12–14), that postmenopausal hormone replacement therapy may inhibit cataractogenesis in women. This protective effect in the lens is one more benefit in a range of advantages, spanning maintenance of bone mineral density to protection against cardiovascular disease (50, 51), all of which might be attributed to estrogen therapy.

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Strain Differences in Vaginal Responses to the Xenoestrogen Bisphenol A

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Bisphenol A (BPA) is the monomer component of polycarbonate plastics and epoxy resins; human exposure derives from leachate in foodstuffs packaged in certain plastics or from epoxy-based dental appliances. BPA stimulates prolactin secretion in Fischer 344 (F344) rats but not in Sprague-Dawley (S-D) rats. The present studies were performed to determine if another classic estrogen target tissue, the rat vagina, responds to BPA in a strain-specific manner. In F344 rats BPA increased DNA synthesis in vaginal epithelium with a median effective dose (ED_{50}) of 37.5 mg/kg body weight; DNA synthesis was not stimulated in S-D rats by any dose tested. Clearance of ^{3}H-BPA from blood followed the same time course in both strains of rats, with a half-life of 90 min. Scatchard analysis of [^{3}H]estradiol binding showed no strain differences in concentration or affinity of the vaginal estrogen receptor. BPA increased the level of mRNA for the immediate early gene, c-*fos*, with similar dose–response curves in both rat strains. Thus, F344 and S-D rats exhibit differences in sensitivity to BPA at the level of cell proliferation in the vaginal epithelium. However, metabolic clearance of BPA and the early events that lead to the proliferative response, receptor-ligand interaction and induction of immediate early genes, show no strain differences. These observations suggest that differences in intermediate effects must account for the difference in sensitivity of the proliferative response to the xenoestrogen. Furthermore, these results point to the need for caution in choosing a suitable end point and animal model when seeking to test the estrogenic effects of xenobiotics. *Key words*: bisphenol A, cell proliferation, c-*fos*, dose response, rat, vagina, xenoestrogen. *Environ Health Perspect* 108:243–247 (2000). [Online 8 February 2000] *http://ehpnet1.niehs.nih.gov/docs/2000/108p243-247long/abstract.html*

Bisphenol A (BPA) is the monomer component of polycarbonate plastics. It behaves as a weak estrogen in classic bioassays, including cellular proliferation and cornification of the vaginal epithelium (*1,2*) and pituitary prolactin secretion (*3*) in the ovariectomized rat, and it produces estrogen-like effects in rat mammary gland (*4*) and developing mouse prostate (*5*). Nearly 2 billion pounds of BPA are manufactured annually in the United States (*6*). Although it is almost entirely in its polymerized form, the BPA monomer finds its way into foodstuffs as a leachate of plastic packaging (*7,8*). Also, humans can be exposed to BPA from certain dental appliances (*9,10*). Because of its estrogenic activity, there is concern over human exposure to BPA.

When assessing the biologic activity of a putative estrogenic compound in animal model systems, it is important to consider species and strain differences. We previously found that BPA increased blood prolactin levels in Fischer 344 (F344) but not Sprague-Dawley (S-D) rats (*3*). Such a finding is congruent with the earlier observation that estrogens stimulate growth of the pituitary through replication of the prolactin-secreting cells in F344 rats but not in outbred rat strains (*11–13*). We also found that the uterine epithelium of F344 rats but not that of S-D rats exhibited a hypertrophic response to BPA administered in low doses (*2*). These observations raise the question of whether there is a general difference between strains in responsiveness of estrogen target tissues to BPA.

The vaginal epithelium is a classic target tissue for studying the estrogenicity of a compound. Estrogens induce proliferation of the cells in the basal layer, thereby producing a multilayered, thick stratified epithelium; with continued estrogen stimulation the superficial layer of cells becomes keratinized (*14*). More recently, it was shown that the immediate early genes, c-*fos* and c-*jun*, are induced by estrogen in target tissues (*15–18*). The induction of these genes is believed to play a key role in cell proliferation (*19,20*). In the present study, we determined the potency of BPA to induce immediate early gene expression and DNA synthetic activity in the vaginal epithelium of F344 and S-D rats.

Materials and Methods

Animals. All procedures performed on animals followed the NIH *Guide for the Care and Use of Laboratory Animals* (*21*) and were approved by the Indiana University Animal Care and Use Committee. F344 and S-D rats were supplied at 6–8 weeks of age by Harlan Sprague-Dawley (Indianapolis, IN). One week after arrival, animals were ovariectomized under ketamine anesthesia; they were subjected to experimental manipulation 3 weeks later. BPA (Aldrich Chemical Co., Milwaukee, WI) and 17β-estradiol (E_2; Sigma Chemical Company, St. Louis, MO) were dissolved in ethanol and diluted in sesame oil. Compounds were injected intraperitoneally (ip) in 50 µL of solution. We injected BPA at 0.2–150 mg/kg body weight (bw) and E_2 at 0.02–2.0 µg/kg bw.

Bromodeoxyuridine immunostaining. Animals were injected with bromodeoxyuridine (BrdU; 100 mg/kg bw in 500 µL saline, ip) and killed 1 hr later. Tissue taken from treated animals was fixed in ethanol–chloroform–acetic acid (60:30:10, vol/vol/vol) at room temperature for 24 hr. The tissue was embedded in paraffin and cross-sections (6 µm) were prepared. After deparaffinization, slides were treated with 0.6% H_2O_2 in methanol for 30 min and then subjected to acid hydrolysis by incubation in 1 N HCl for 8 min, followed by 0.0125 M borate buffer (pH 8.5) for 15 min. Sections were incubated in 5% horse serum to block nonspecific immunostaining and then incubated in anti-BrdU antibody (Becton Dickson, San Jose, CA) overnight, followed by incubation in a biotinylated secondary antibody and avidin-biotinylated peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogen. We counted the BrdU-labeled epithelial cells and determined the length of the underlying basement membrane using image analysis software (IPLab Spectrum; Signal Analytics, Vienna, VA). Results were expressed as the number of labeled cells per millimeter; using several sections from each animal, we counted labeled cells along a sufficient length of basement membrane to include > 1,000 basal epithelial cells.

BPA clearance. To determine the relative rate of clearance of radiolabeled BPA from blood after a single bolus intravenous injection, we anesthetized two animals (150 g bw) of each strain and injected a tail vein

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with 25 µCi (2.9 µg/kg bw) ^{3}H-BPA (13 Ci/mmol; Moravek Biochemicals, Brea, CA) in 250 µL balanced salt solution containing 5% bovine calf serum. Blood was collected into heparinized capillary tubes from a separate tail vein at intervals between 5 and 120 min. After centrifugation, 50 µL plasma was added to a scintillation vial and counted. The half-life of the radiolabeled compound was determined from samples taken between 30 and 120 min.

Estrogen receptor assays. Vaginas from ovariectomized adult rats were homogenized and centrifuged to prepare a cytosol, as described previously (*22*). Aliquots of the cytosol were incubated with increasing concentrations of [^{3}H]estradiol (Amersham, Arlington Heights, IL) with or without a 100-fold molar excess of diethylstilbestrol (Sigma Chemical Company). Bound and free steroid were separated by the dextran-coated charcoal method (*22*).

RNA isolation and RNase protection assays for c-fos. Total RNA was isolated from vagina by homogenizing the tissues in Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNase protection assays (RPAs) were performed on 5 µg RNA from each specimen using a kit (RPA II; Ambion, Austin, TX) according to the manufacturer's instructions. We derived RNA probes from c-*fos* cDNA as described earlier by Nephew et al. (*23*); the plasmid was linearized with the restriction enzyme *Nco*I. Antisense riboprobes were generated from linearized templates using the MAXIscript kit (Ambion), T_7 RNA polymerase, and ^{32}P-uridine triphosphate (New England Nuclear, Boston, MA) according to the kit instructions. Cyclophilin mRNA levels were determined using riboprobe derived from the cDNA template pTRI-cyclophilin (Ambion). After incubation of RNA samples with the radiolabeled riboprobe and RNase, the protected riboprobe was separated on a polyacrylamide gel (6%). The gel was dried and exposed to X-ray film (Kodak X-Omat, Sigma Chemical Company). The X-ray film was analyzed on a GS-670 Imaging Densitometer (BioRad, Hercules, CA). The mRNA levels for c-*fos* were normalized against the level of cyclophilin mRNA by dividing the optical density (OD) of the autoradiographic band for c-*fos* by the OD of the corresponding cyclophilin band of each specimen.

Statistical analysis. The number of BrdU-labeled cells per millimeter or the mRNA levels (arbitrary units of the OD ratios) were analyzed by analysis of variance (ANOVA) across all doses of BPA or E_2, comparing the mean at each dose against the untreated control by Fisher's protected least significant difference (PLSD) test.

Results

In an earlier study (*2*), we determined that the maximum DNA synthesis in vaginal epithelium occurred 20 hr after estrogenic stimulation; therefore, we used this time to determine the dose–response effects of BPA in F344 and S-D rats. The DNA synthetic response to BPA in the vaginal epithelium was dramatically different between the two strains of rats. In F344 rats there was a clear dose response, with a statistically significant increase occurring at 37.5 mg/kg; this dose also corresponds to the approximate median effective dose (ED_{50}) for this effect (Figure 1). However, in S-D rats there was no effect of BPA on vaginal DNA synthesis. In contrast to BPA, there was no strain difference in the vaginal DNA synthetic response to E_2. Although there was a slight difference in the magnitude of the maximal effect (the S-D rats had a higher number of labeled cells), the ED_{50} for E_2 was approximately the same in each strain (Figure 2). It was also apparent that the maximal effect of BPA in F344 rats was approximately one-third of the maximal effect of E_2 (compare Figures 1 and 2).

A simple explanation for the strain difference might be a difference in the rate of metabolic clearance of injected BPA. To test this, animals received an intravenous injection of ^{3}H-BPA and the amount of radioactivity remaining in the blood was determined at various times thereafter, without regard to the proportions of parent compound or metabolite present. Disappearance of radiolabeled BPA was biphasic. After a rapid distribution phase, there was a sustained loss of compound exhibiting a half-life of approximately 90 min for each strain (Figure 3). In two of the animals, approximately one-half of the injected dose of radiolabel was found in the urine that remained in the urinary bladder at the end of 2 hr. Thus, there is a rapid clearance of BPA from the blood, and there does not appear to be any strain difference in this parameter.

The difference in sensitivity to BPA might be explained by a difference in the tissue

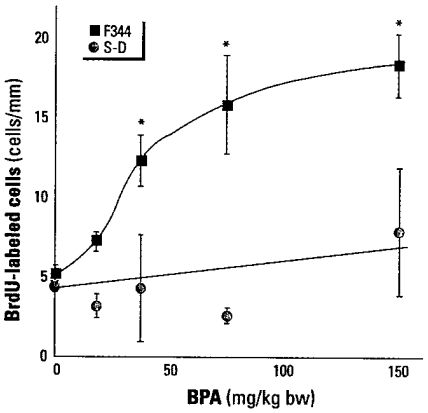

Figure 1. DNA synthetic response to BPA in vaginal epithelia of F344 and S-D rats. Ovariectomized adult rats were treated with BPA at the indicated doses and with BrdU 20 hr later. Values shown are mean ± SE; n = 3–5. F344 data from Steinmetz et al. (*2*). *$p < 0.05$ vs. control.

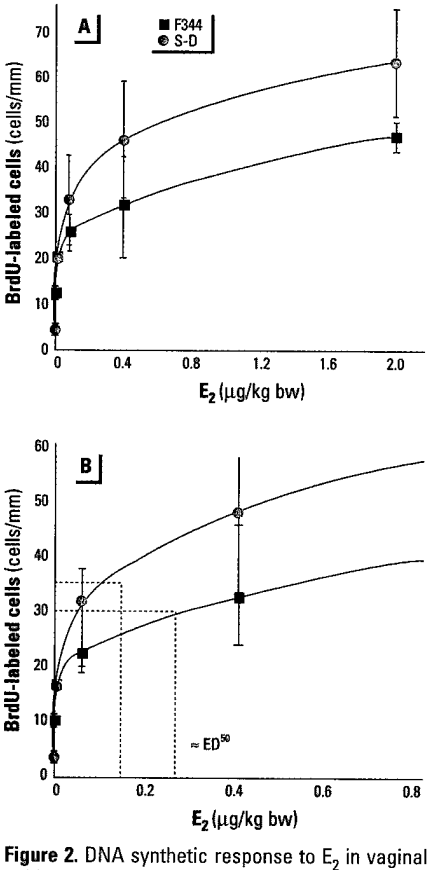

Figure 2. DNA synthetic response to E_2 in vaginal epithelia of F344 and S-D rats. (*A*) DNA synthetic dose response. (*B*) Expanded view of the low end of the dose–response curve to allow estimation of the ED_{50} values. Values shown are mean ± SE; n = 3–5. Ovariectomized rats were treated with the indicated dose of E_2 and with BrdU 20 hr later.

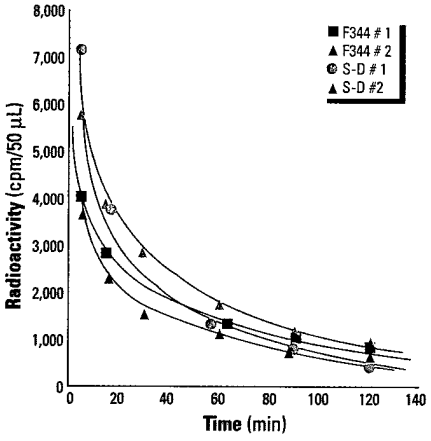

Figure 3. Plasma levels of radioactivity following intravenous injection of ^{3}H-BPA in two animals of each strain. Details are provided in "Materials and Methods."

concentration of estrogen receptor (ER) between rats. To examine this possibility, we analyzed the ER content of vaginal tissue taken from each strain. Scatchard analysis of [^{3}H]estradiol binding showed that there were no differences in concentration or affinity of receptors between the strains (Figure 4). Combining data from four such analyses, ER isolated from F344 rats had a K_d of 0.13 ± 0.023 nM (mean ± SE) and a maximum binding capacity of 266 ± 52.4 fmol/mg protein (mean ± SE), whereas ER from S-D rats had a K_d of 0.14 ± 0.018 nM and a binding capacity of 288 ± 68.4 fmol/mg protein.

The DNA synthetic response occurs several hours after the initial stimulus; this response probably occurs as the result of a cascade of events that are initiated by direct induction of gene transcription. The immediate early gene c-*fos* is part of the primary response to estrogenic stimuli. In a preliminary experiment, animals were treated with 150 mg/kg bw BPA or vehicle and killed at 2, 3, 6, and 24 hr. The RPA of vaginal RNA for c-*fos* showed that BPA induced maximum steady-state levels at 2 hr (Figure 5A); the time course was similar in both strains. We then performed BPA dose–response studies in which animals were sacrificed 2 hr after administration of the xenobiotic. Stimulation of c-*fos* expression by BPA showed no strain difference; both strains of rats responded equally well, exhibiting ED_{50} values at 37.5 mg/kg (Figure 5B).

Discussion

Although the xenobiotic BPA is considered a weak estrogen, there is mounting evidence that it can in fact elicit full estrogenic activity in some bioassay systems. Treatment of immature or adult ovariectomized rats with BPA produced little or no increase in uterine weight (*2,24,25*) and it can partially inhibit the uterotrophic effect of estradiol (*24*), responses typical of a partial estrogen. However, a 3-day treatment of ovariectomized rats with BPA caused the vaginal epithelium to become fully keratinized (*1,2*); this is the response of a full agonist. Also, prolactin secretion was increased to the same extent by estradiol or BPA, but pituitary growth was not induced (*3*). Induction of c-*fos* expression by BPA reached a magnitude, and followed a time-course, that would be expected for a natural estrogen (*15–18*). In a preliminary study, we also found that BPA induced expression of c-*jun* in both strains of rats, with steady-state levels reaching maximum at 2–3 hr after treatment (data not shown). Likewise, the time-course of the DNA synthetic response in the F344 vagina following a single injection of BPA is similar to that produced by natural estrogens (*26,27*). In studies on estrogenic effects in non-reproductive tissues, Dodge et al. (*25*) found that BPA lowered cholesterol levels but did not protect against bone loss in ovariectomized rats. Thus, depending on the end point and the animal model under study, BPA may be considered a partial or a full estrogen agonist *in vivo*. On the other hand, BPA produces full estrogenic effects in breast cancer cells *in vitro* (*9*). Perhaps the recently coined terminology "selective ER modulator" (SERM) (*28*) is a more appropriate characterization of the activity of BPA.

The ED_{50} values for BPA effects, as determined in this study, were approximately 40 mg/kg bw. It is unlikely that such a high dose would be encountered in a single environmental exposure. However, the apparent *in vivo* potency of BPA is dependent on the route of administration and dosing schedule. When BPA was applied by subcutaneous insertion of a continuous release capsule that delivered approximately 300 µg/kg bw/day, a full estrogenic response was produced in the vaginal epithelium of F344 rats (*2*). Thus, the biologic potency of this compound may depend on dosage, dosing schedule, and route of administration.

Biologic potency also depends on the metabolic clearance rate of a compound, and this may differ between the two strains of rats. To address this, we investigated the pharmacokinetics of a bolus injection of BPA. Knaak and Sullivan (*29*) showed that over the first 24 hr after BPA was administered orally to rats, > 80% of the administered material was excreted as a glucuronide in the urine or eliminated as free compound and a hydroxylated metabolite in the feces. Also, 8 days after administration, no BPA was detected in the animals (*29*). In the present study, circulating BPA had a half-life of approximately 90 min. Although this simple analysis did not determine whether the radiolabel remaining in the blood was authentic starting material, it nonetheless indicates that there is a rapid clearance of BPA from the blood. Furthermore, the rate of clearance was similar in F344 and S-D rats, indicating that strain-specific metabolic clearance does not play a role in establishing the difference in the biologic responses. Further studies are required to determine additional pharmacokinetic parameters under the condition of a continuous administration regimen.

Our results show that the F344 inbred rat strain is more sensitive to BPA induction of DNA synthesis in vaginal epithelium than the outbred S-D rat strain. Furthermore, this strain difference appears to be specific to the weakly estrogenic compound BPA, as there was no difference between strains in sensitivity to E_2 stimulation. Yet, the initial events that lead to the

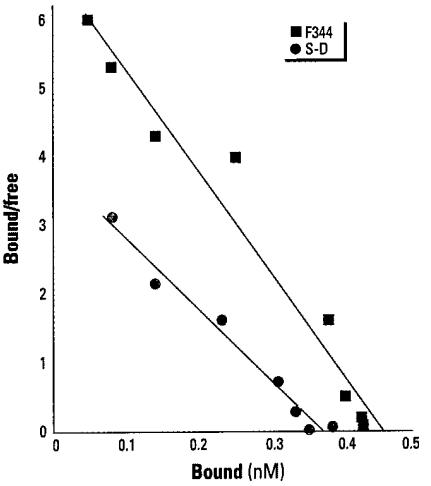

Figure 4. Scatchard analysis of vaginal estrogen receptor. Cytosol was prepared from vaginas of F344 and S-D rats. A standard saturation analysis of binding activity for [^{3}H]estradiol was performed using the dextran-coated charcoal method. A typical Scatchard plot from one of four such assays is shown.

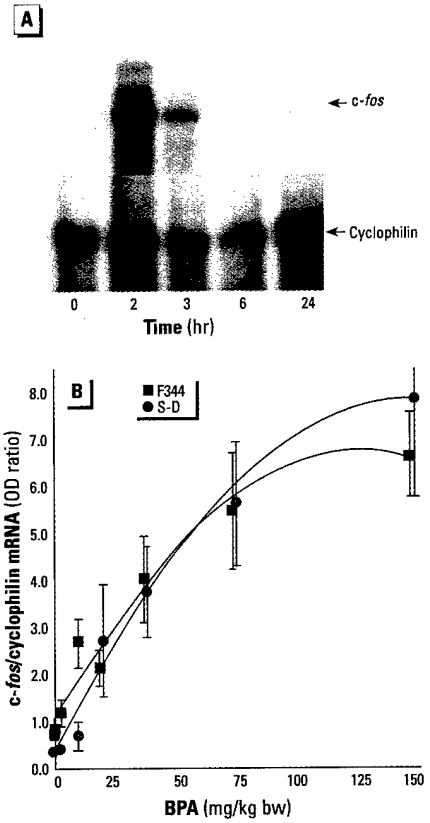

Figure 5. RPA determinations of c-*fos* mRNA steady state. (*A*) An autoradiogram of the RPA bands for c-*fos* and cyclophilin at various times after BPA treatment in F344 rats. (*B*) Steady-state levels of c-*fos* were determined 2 hr after BPA treatment in F344 and S-D rats. The intensity (OD) of the c-*fos* band was normalized against the OD of the corresponding cyclophilin band. Values shown are mean ± SE; $n = 4$.

DNA synthetic response, ER binding and immediate early gene transcription, show no strain differences that account for the difference in sensitivity to BPA. Although BPA induced expression of immediate early genes in S-D rats, this expression was not sufficient to produce the proliferative response; this is similar to a single dose of either of the weak estrogens estriol or 16α-E_2, which was able to induce expression of immediate early genes but did not induce the growth response in rat uterus (*30,31*). In a preliminary study, we found that three daily injections of estriol produced a uterine growth response and that this treatment was more effective in F344 rats than in S-D rats (*32*).

Our observations suggest that the strain difference is due to a delayed, or intermediate, effect rather than a primary response mechanism. Lanahan et al. (*33*) described a set of "delayed early genes" whose expression is induced by growth stimuli, but only after several hours. Using a short-acting estrogen, 16α-E_2, Stack and Gorski (*34*) showed that stimulation of DNA synthesis in the rat uterus is positively correlated to the rate of protein synthesis at 12 hr after an injection of estrogen. Cheng and Pollard (*35*) showed that uterine expression of c-*ras*H and ornithine decarboxylase increased 6–12 hr after estradiol treatment. Dean and Sanders (*36*) suggested that there are two classes of genes which respond to estrogenic stimuli in a delayed manner: the secondary response genes that are dependent on the products of the early primary response genes for their stimulation, and the delayed primary response genes that are dependent on a direct interaction of steroid receptor with the gene's promoter and concomitant enhancement by a product of the early primary response gene. It may be that the delayed response genes stimulated by E_2 in both S-D and F344 rats are not induced by BPA in the S-D rat but are induced by BPA in the F344 rat; this possibility requires further investigation.

Strain differences in response to estrogenic stimuli have been previously demonstrated. Gorski and co-workers (*11–13*) found that the potent estrogens diethylstilbestrol or E_2 induce an overgrowth of lactotropes in the pituitary glands of F344 rats but not in those of outbred strains of rats. Recently, Spearow et al. (*37*) found dramatic strain differences in the susceptibility of mice to estradiol-induced disruption of testicular development. Others have shown differences in the efficacy of E_2 in stimulation of uterine DNA synthesis between strains of mice (*38,39*). Our data extend these observations to the vaginal response in rats and point out strain differences in sensitivity to a weak estrogen, BPA. This type of difference must be taken into account when utilizing the classic vaginal response model for assessment of estrogenic activity of test compounds.

The genetic parameters that are responsible for strain differences in estrogen sensitivity or efficacy are largely unknown. However, Roper et al. (*40*) recently reported that several genetic quantitative trait loci may be partly responsible for the varied efficacy of E_2 to induce uterine growth in different strains of mice. Similarly, Wendell and Gorski (*13*) identified five quantitative trait loci that genetically account for more than half of the difference between estrogen-induced growth of pituitary glands in F344 rats and Brown Norway rats, but these chromosomal loci do not correspond to those that account for strain differences in uterotrophic responses in mice (*40*). At present, although a number of genes located within the quantitative trait loci are known, the specific genes responsible for these strain differences have not been identified (*40*). There were no strain differences in estrogenic stimulation of several oncogenes or angiogenic factors in the pituitary gland; therefore, these genes are unlikely to mediate this effect (*41*). Similarly, we found that the immediate early genes c-*fos* and c-*jun* had no apparent role in establishing the strain difference to BPA stimulation in the vagina. Also, we have found that the ERs of each strain of rats have the same characteristic binding affinity and tissue concentration, ruling out alterations at this level as the simple explanation. As mentioned above, it may be that a later event, such as modulation of an intermediate gene, accounts for the strain differences. Identification of the genetic traits responsible for strain differences in sensitivity to the action of weak estrogens in rodents may yield valuable insight into the causes of varied susceptibilities to xenoestrogen action in humans.

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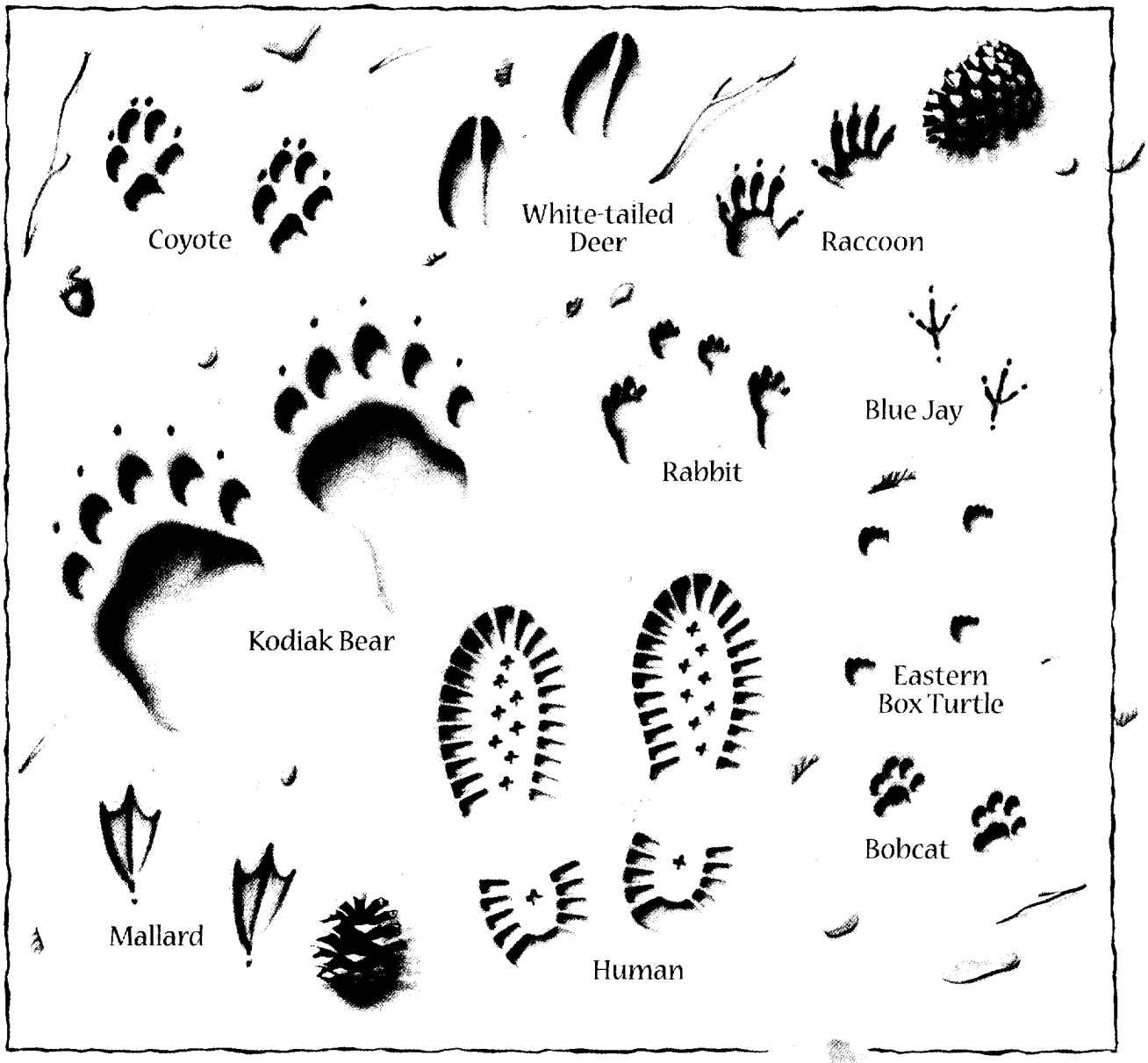
WHICH ONE OF THESE CREATURES CAN EAT UP A WHOLE FOREST OVERNIGHT?



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Environmentally Relevant Xenoestrogen Tissue Concentrations Correlated to Biological Responses in Mice

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The effects of xenoestrogens have been extensively studied in rodents, generally under single, high-dose conditions. Using a continuous-release, low-dose system in ovariectomized mice, we correlated the estrogenic end points of uterine epithelial height (UEH) and vaginal epithelial thickness (VET) with concentrations of two organochlorine pesticide isomers in fat and blood. Silastic capsules containing a range of doses of either β-hexachlorocyclohexane (β-HCH) or *o,p′*-dichlorodiphenyltrichloroethane (*o,p′*-DDT) were implanted subcutaneously, and animals were killed after 1 week. Average blood levels achieved by the various doses were 4.2–620 ng/mL for *o,p′*-DDT and 5.0–300 ng/mL for β-HCH. Fat concentrations of *o,p′*-DDT and β-HCH correlated linearly to blood levels (*o,p′*-DDT, r^2 = 0.94; β-HCH, r^2 = 0.83). Fat concentrations (nanograms per gram of tissue) were higher than blood concentrations (nanograms per milliliter) by 90 ± 5- and 120 ± 9-fold (mean ± SE) for *o,p′*-DDT and β-HCH, respectively. The VET ranged from 12 ± 0.9 μm in controls to 114 ± 8 μm in treated animals, and was correlated to blood levels of either treatment compound. The UEH ranged from an average of 7.7 ± 0.3 μm in controls to 26 ± 2 μm in high-dose *o,p′*-DDT-treated animals. The UEH was also correlated with β-HCH concentration, but it plateaued at approximately 11 μm at the highest doses. The lowest blood concentrations that produced statistically significant increases in VET or UEH were 18 ± 2 ng/mL *o,p′*-DDT and 42 ± 4 ng/mL β-HCH. These values are within the same order of magnitude of blood concentrations found in some human subjects from the general population, suggesting that human blood concentrations of these organochlorines may reach estrogenic levels.
Key words: β-HCH, dose response, histology, mouse, *o,p′*-DDT, uterine epithelium, vaginal epithelium, xenoestrogen. *Environ Health Perspect* 108:973–977 (2000). [Online 7 September 2000]
http://ehpnet1.niehs.nih.gov/docs/2000/108p973-977ulrich/abstract.html

Although banned in most industrialized nations, organochlorine (OC) pesticides are still used in Third World countries and are ubiquitous and persistent pollutants (*1*). Dichlorodiphenyltrichloroethane (DDT) is infamous as an endocrine disruptor in birds (*2*), and the *o,p′*- isomer of DDT, which constitutes approximately 15% of the technical mixture, is estrogenic *in vitro* and *in vivo* (*3*). Another estrogenic pesticide residue is the β-isomer of hexachlorocyclohexane (HCH), which constitutes 7–12% of technical HCH (*4*). β-HCH has several toxicologic effects in addition to estrogenic activity; also, it preferentially accumulates in the fat of biota and biomagnifies as it moves through the food web (*4*). Because virtually all humans have been exposed to these compounds, the concern over their potential health effects continues to grow. Recent epidemiologic studies have been controversial regarding the relationship of OC blood levels and human disease (*5–16*). The debate is likely to continue because there are no "unexposed" cohorts for comparison, and exposure information is difficult to obtain. For these reasons, we must rely on experimental animal studies as a guide to determine rational safety levels for humans.

Many studies, both *in vivo* and *in vitro*, probe a variety of effects caused by these OC pesticides. Although β-HCH does not competitively bind to the estrogen receptor (*17,18*), it does produce a number of estrogen-like responses: it stimulates proliferation and increases synthesis of progesterone receptors in cultures of human breast cancer cells (*17,18*), and it produces moderate uterotrophic effects in the rodent uterus (*18,19*). In contrast, *o,p′*-DDT does competitively bind to the estrogen receptor (*18,20,21*), and it produces a range of estrogenic responses (*18–23*). However, previous *in vivo* studies have used only a few or very high doses of these chemicals, and only the doses applied, not the resulting blood concentrations, were reported.

The present report describes an *in vivo* experiment designed to correlate the estrogenic end points of uterine epithelial cell height (UEH) and vaginal epithelial thickness (VET) with blood and fat levels of *o,p′*-DDT and β-HCH in ovariectomized mice. To simulate the effect of chronic low-level exposure seen in humans, a low dose, continuous release system of Silastic capsules was employed. A wide range of doses was administered, and concentrations of treatment compound were measured in the blood and fat of the mice. This is the first study to correlate blood and tissue concentrations of these compounds to estrogenic response in laboratory animals. These results are important to the discussion of safe exposure for humans and wildlife because they allow direct comparisons between blood levels and biological responses.

Materials and Methods

Mouse treatment. All procedures performed on animals followed the NIH *Guide for the Care and Use of Laboratory Animals* (*24*), and were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. Three weeks after adult female CD-1 outbred mice were ovariectomized, animals were treated with *o,p′*-DDT or β-HCH over a dose range of approximately 32-fold by subcutaneous insertion of Silastic capsules containing crystalline treatment compound. A positive control group consisted of animals that received a Silastic capsule containing 20 mg estrone (Sigma Chemical Co., St. Louis, MO). Negative control animals were treated with an empty capsule. Treatment groups consisted of five animals per dose. Treatment capsules were made from Silastic tubing (1.6 mm i.d., 3.2 mm o.d., and 14 mm length; Konigsberg Instruments, Pasadena, CA) and sealed at each end with Silastic cement. Each capsule contained approximately 20 mg crystalline material. Low doses were achieved by inserting a single capsule containing treatment compound mixed with crystalline cholesterol; mixtures were prepared to make "dilutions" of one-half, one-fourth, and one-eighth. The higher doses were achieved by inserting two capsules of the one-half dilution, or one, two, or four capsules containing only test compound. Each Silastic capsule was implanted subcutaneously through a 5-mm slit on the back. After 1 week of treatment, animals were anesthetized and exsanguinated by heart puncture. The uterus and vagina of each animal was removed for histomorphometric determination of the estrogenic effect. Blood serum and intraperitoneal fat samples were

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frozen and saved for analysis of *o,p'*-DDT or β-HCH concentrations.

Histomorphometrics. Uterine and vaginal tissues were fixed in neutral formalin and processed for paraffin sections. Tissue sections (6 μm) were stained with hematoxylin and eosin. Cross-sections were examined under a light microscope (Nikon Optophot; Nikon, Fryer Co., Huntley, IL) that was interfaced with a Macintosh PowerPC computer (Apple Computer, Inc., Cupertino, CA) through a Sony 3CCD color video camera (Sony, The Systems Group, Ann Arbor, MI). The height of the uterine epithelium and the thickness of the vaginal epithelium were determined using an image analysis program (IPLab Spectrum, Signal Analytics, Vienna, VA).

Blood extractions. Three or four of the five blood samples from each dose were processed for *o,p'*-DDT or β-HCH concentration analysis. Approximately 100 μL mouse serum was extracted three times with 20 mL hexane (EM Scientific, Gibbstown, NJ). To increase the volume of the aqueous phase, 1 mL of 90% formic acid (Fisher Scientific, Fair Lawn, NJ) was added to the serum. We used γ-HCH as an internal standard for the mice treated with β-HCH and *p,p'*-DDT for the mice treated with *o,p'*-DDT (Accustandard, New Haven, CT). The resulting aqueous phase was extracted with 1 min of vortex mixing and 1 min of centrifugation for each aliquot of hexane.

Fat extractions. Fat tissues (0.25 g) were ground with 15 g of 10–60 mesh anhydrous sodium sulfate (Fisher Scientific) and spiked with the appropriate internal standard as above. These mixtures were then Soxhlet extracted for 24 hr in 300 mL 50% acetone in hexane (EM Scientific).

Quality control. With each set of six samples, we also extracted a matrix spike and either a matrix blank or a glassware blank. All quality control samples underwent the same cleanup procedure as the samples. The matrix spike contained a known amount of the target analyte similar in concentration to what we expected in the samples. Spike recovery averaged 104 ± 7%, and sample concentrations were not corrected for recovery. No target compound was found in any type of blank; therefore, blank correction was unnecessary.

Lipid analysis and removal. For fat samples only, lipid analysis was performed gravimetrically in duplicate. The removal of lipids was performed using a gel permeation chromatography column. The glass column (2.5 cm × 100 cm) was packed with SX8 Bio-Beads (Bio-Rad Laboratories, Hercules, CA) and eluted with 60% cyclohexane in dichloromethane (EM Scientific) at a flow rate of 10 mL/min through the column. The lipids were eluted in the first 200 mL fraction, whereas the HCHs and DDTs were eluted in the following 400 mL fraction.

Silica column cleanup. Sample extracts were reduced to approximately 1 mL by rotary evaporation and were exchanged into hexane as necessary. Fractionation was performed to remove possible interferences from the samples. The extracts were run through a silica (grade 923; Grace Davison, Baltimore, MD) column (1.25 cm diameter) consisting of glass wool, 20 cm of silica (1% HPLC-grade water deactivated), and 1 cm sodium sulfate. For both compounds, we collected three fractions of 75 mL each. HCH samples were fractionated with hexane, 50% dichloromethane in hexane, and dichloromethane, whereas DDT samples were fractionated with

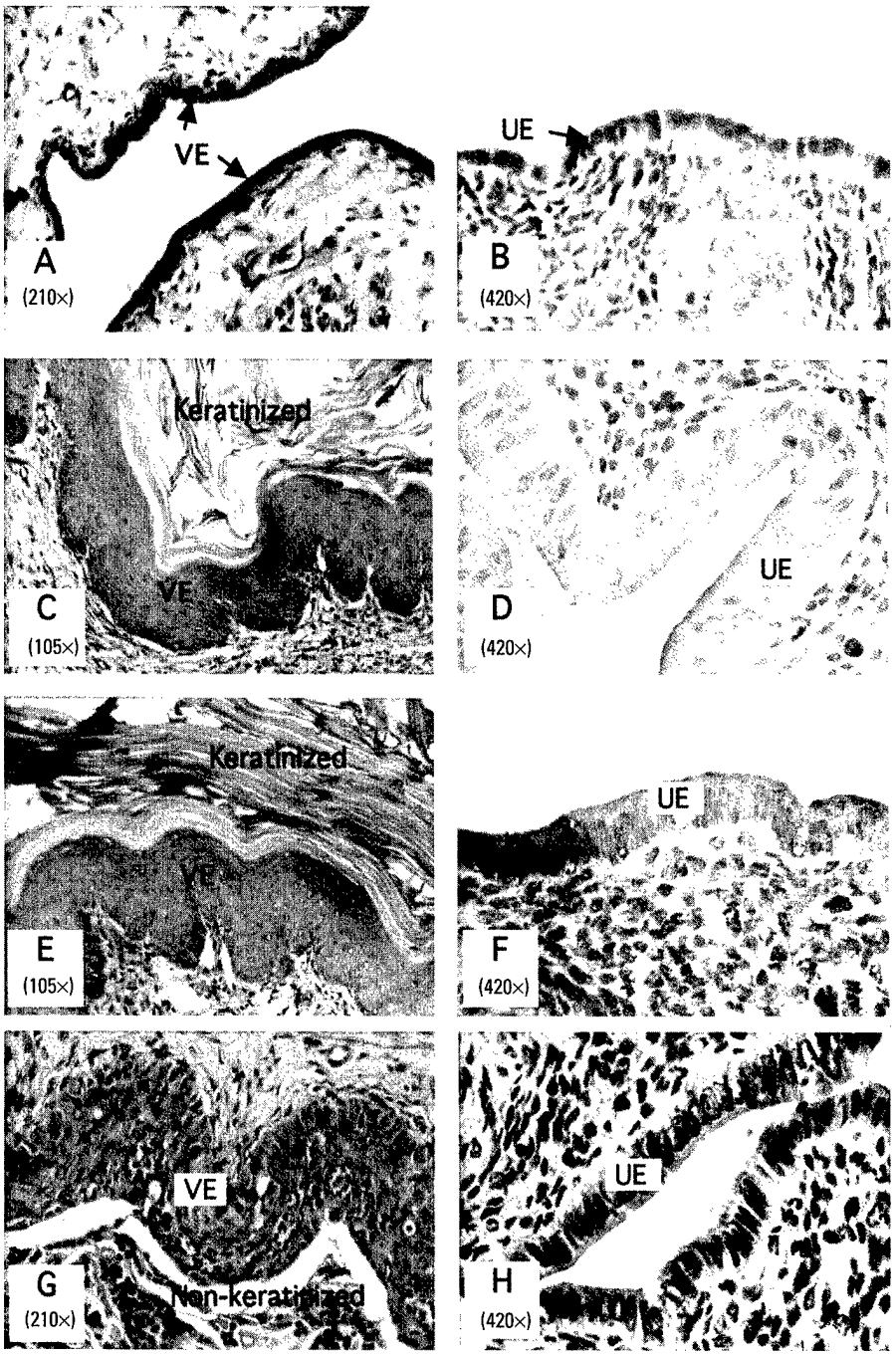

Figure 1. Histology of vagina and uterus in treated mice. Shown are representative histologic sections of vagina (*A, C, E, G*) and uterus (*B, D, F, H*) from mice treated for 1 week with an empty implant (*A, B*), an estrone implant (*C, D*), the highest dose of *o,p'*-DDT (*E, F*), or the highest dose of β-HCH (*G, H*). The final magnification is shown in each panel. Darker cells labeled VE and UE are the measured vaginal epithelium and uterine epithelium, respectively. In the estrone-treated (*C*) and *o,p'*-DDT-treated animals (*E*), there were several layers of keratinized cells in the superficial layers of the vaginal epithelium.

hexane, 20% dichloromethane in hexane, and dichloromethane. The HCHs and DDTs were eluted in the second fraction. All three fractions were reduced by rotary evaporation and solvent exchanged into hexane, if necessary. The first and third fractions were frozen and stored, but not analyzed. The second fraction was further reduced to approximately 50 µL by a gentle stream of nitrogen and transferred into an autosampler vial with two to three rinses of hexane.

Instrumental parameters. The samples were analyzed on a Hewlett Packard 5890A gas chromatograph (Hewlett Packard, Palo Alto, CA) with an electron capture detector. The carrier gas was hydrogen (80 mL/min split vent, 2 mL/min on column) and the makeup gas (25 mL/min) was nitrogen (Gas Tech, Hillside, IL). Injections of 1 µL were made by an autosampler in the splitless mode, and the purge flow (2 mL/min) was opened after 3 min. We used a 60-m DB5 column (J&W Scientific, Folsom, CA), with 250 µm i.d. and a film thickness of 0.1 µm, for separation. The temperature program for the DDTs was 40°C for 1 min, ramped to 130°C at 30°C/min, ramped to 241°C at 3°C/min, and ramped to 285°C at 30°C/min, with a 10-min hold for a total analysis time of 52 min. The HCH temperature program was 50°C for 1 min, ramped to 130°C at 20°C/min, ramped to 160°C at 1°C/min, and ramped to 290°C at 30°C/min, with a 1-min hold for a total analysis time of 40 min.

Statistical analysis. We used linear regression analysis to compare blood and fat concentrations of a compound to each other and to compare biological response (VET or UEH) against blood or fat concentrations of a test compound. We used analysis of variance (ANOVA) and the Dunnett Test to evaluate responses at each dose relative to control (empty capsule treatment) response values. Because variance increased with increasing dosage, response values were log transformed before applying ANOVA.

Results

Estrogenic responses. The effects of the highest doses of each compound are shown in Figure 1. *o,p'*-DDT increased VET to the same extent as estrone. In addition, the superficial layers of the vaginal epithelium were keratinized in animals treated with either estrone or *o,p'*-DDT. Uterine epithelial cell height was also increased maximally by *o,p'*-DDT. The effects of β-HCH were less than those produced by estrone. The highest dose of β-HCH produced keratinized vaginal epithelium in only two of the five animals in this group. However, there was an apparent proliferative effect in the other three animals, as evidenced by the increased tissue thickness.

Blood and fat concentrations. Blood concentrations of β-HCH and *o,p'*-DDT averaged from 5.0 to 300 ng/mL and 4.2 to 620 ng/mL, respectively. Concentrations of these compounds were much higher in fat, 1,300–42,000 ng/g tissue and 270–77,000 ng/g tissue for β-HCH and *o,p'*-DDT, respectively. Figure 2 shows the strong linear relationship between blood and fat concentrations for each compound, with fat concentrations related to blood levels by coefficients of 90 ± 5 and 120 ± 9 (SE) for *o,p'*-DDT and β-HCH (r^2= 0.94 and r^2 = 0.83), respectively.

Histomorphometrics. Control animals had VET and UEH values of 11.7 ± 0.94 µm and 7.7 ± 0.32 µm (mean ± SE), respectively. VET was increased 10-fold by the highest dose of *o,p'*-DDT and 5-fold by β-HCH at its highest dose; however, the high dose of β-HCH resulted in blood levels that were approximately one-half those achieved by the high dose of *o,p'*-DDT (Table 1). Thus, on a nanogram per milliliter basis, *o,p'*-DDT and β-HCH were equally effective in vaginal epithelium. There was a linear correlation between log blood concentration of either compound and log VET or log UEH (Figure 3A-D); in each case the correlation was statistically significant ($p \leq 0.002$ for VET and UEH against either *o,p'*-DDT or β-HCH). As expected from the strong correlation between blood and fat concentrations, dose–response curves for VET and UEH showed similar trends when plotted against fat levels (not shown). Thus, only the blood values are used for further discussions of estrogenic responses.

For the purpose of comparison, animals dosed with estrone had a VET (mean ± SE) of 80 ± 7 µm and a UEH of 25 ± 3 µm (Table 1). Mice with *o,p'*-DDT blood concentrations > 260 ng/mL had VET and UEH responses as high or higher than the estrone-induced effect. None of the β-HCH treatments caused responses as high as those achieved in estrone-treated animals; in fact, the UEH response to β-HCH reached a

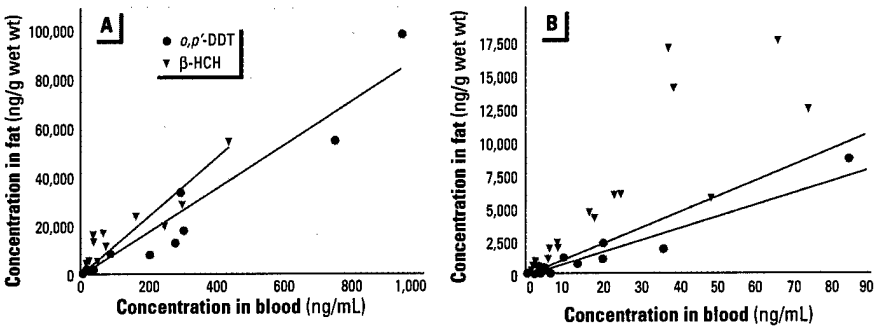

Figure 2. *o,p'*-DDT and β-HCH concentrations in fat and blood of (*A*) 20,000–100,000 ng/g wet wt and 0–1,000 ng/mL, respectively, and (*B*) 2,500–17,500 ng/g wet wt and 0–90 ng/mL, respectively. Linear relationships were forced through the origin. The slope of the line (ng/g fat)/(ng/mL blood) indicates a magnification factor from blood to fat of 120 ± 9 for β-HCH and 90 ± 5 for *o,p'*-DDT (mean ± SE). Correlation coefficients were statistically significant for both β-HCH (r^2 = 0.83; $p < 0.001$) and *o,p'*-DDT (r^2 = 0.94; $p < 0.001$).

Table 1. Blood concentrations and biological end points.

Treatment group	Blood concentration (ng/mL)	UEH (µm)	VET (µm)
Control	0	7.74 ± 0.32 (5)	11.7 ± 0.94 (5)
Estrone	ND	25.5 ± 3.5 (5)**	79.6 ± 6.6 (5)**
o,p'-DDT			
A (1/8)	4.4 ± 0.61 (4)	7.88 ± 0.62 (4)	14.3 ± 2.5 (4)
B (1/4)	5.2 ± 0.91 (3)	8.93 ± 0.22 (3)	14.7 ± 0.87 (3)
C (1/2)	23 ± 13 (2)	9.15 ± 0.66 (3)	20.0 ± 1.7 (4)*
D (2 × 1/2)	18 ± 2.3 (3)	12.0 ± 0.95 (5)**	39.7 ± 7.0 (5)**
E (1)	190 ± 40 (4)	11.4 ± 1.6 (4)*	63.9 ± 10 (4)**
F (2)	260 ± 26 (4)	15.7 ± 1.9 (4)**	85.3 ± 21 (4)**
G (4)	620 ± 150 (4)	26.0 ± 1.7 (4)**	114 ± 8.4 (4)**
β-HCH			
A (1/8)	5.0 ± 0.97 (3)	8.40 ± 0.61 (4)	12.1 ± 1.7 (4)
B (1/4)	12 ± 3.4 (3)	7.18 ± 0.75 (4)	15.1 ± 1.1 (4)
C (1/2)	22 ± 2.5 (3)	9.03 ± 1.2 (5)	17.6 ± 4.2 (5)
D (2 × 1/2)	42 ± 3.6 (3)	9.97 ± 1.0 (4)	21.9 ± 2.7 (5)**
E (1)	66 ± 3.9 (3)	12.1 ± 0.61 (4)**	38.3 ± 6.1 (4)**
F (2)	170 ± 29 (4)	10.8 ± 0.71 (5)*	38.9 ± 2.9 (5)**
G (4)	300 ± 49 (4)	11.3 ± 0.82 (5)*	58.1 ± 5.2 (5)**

ND, not determined. Mean values of blood serum concentrations, UEH, and VET were determined for each treatment/dosage group. For *o,p'*-DDT and β-HCH, dosage groups are listed as A–G for treatments with a single diluted capsule (1/8, 1/4, 1/2), a single undiluted capsule (1), or multiple capsules (2 × 1/2, 2, 4). Values are presented as mean ± SE; numbers in parentheses are the number of samples analyzed.
*$p < 0.05$. **$p < 0.005$ as compared to control.

plateau of approximately 11 μm for blood concentrations > 66 ng/mL (Table 1).

The doses in this experiment are low enough to observe a no-observed-effect level (NOEL). The lowest-observed-effect levels (LOELs) in VET were detected at blood concentrations of 42 ng/mL and 18 ng/mL for β-HCH and *o,p′*-DDT, respectively (Table 1). Statistically significant increases in UEH were detected at blood concentrations of 66 ng/mL and 18 ng/mL for β-HCH and *o,p′*-DDT, respectively (Table 1).

Discussion

Blood and fat concentrations. There is a strong linear relationship between the blood and fat concentrations for both compounds. For β-HCH, fat levels (nanograms per gram wet wt) were related to blood levels (nanograms per milliliter) by a factor of 120; fat levels of *o,p′*-DDT were related to blood levels by a factor of 90. Similar trends have been observed in humans. In a Canadian study of paired blood and fat concentrations, Mes (*25*) found 43- to 355-fold higher levels of β-HCH in fat and a 33-fold higher level of *o,p′*-DDT in fat. In an Indian study, Ramachandran et al. (*26*) found 35-fold (male) to 38-fold (female) higher levels of β-HCH in fat and 20-fold (female) to 25-fold (male) higher levels of *o,p′*-DDT in fat. The linearity of these relationships allows us to use either blood or fat values in our discussion of estrogenic effects. Because blood levels are more readily available in reports of human exposure, we will focus on the blood levels attained in our experimental system.

Dose–response curves. In general, β-HCH produced a slightly lower response than did *o,p′*-DDT across all blood levels observed. There is a marked difference in the maximal efficacy of these compounds, especially in the uterus. In both the uterus and vagina, *o,p′*-DDT is more potent than β-HCH, producing a significant effect at 18 ng/mL compared to 42 ng/mL required for a significant effect by β-HCH. The observation that the uterine response to β-HCH reached a plateau at higher doses suggests that β-HCH is a compound with differential effects of the vagina and uterus. Nishino and Neumann (*27*) have shown that two estrogen derivatives [8α-estra-1,3,5(10)-triene-1,3,17β-triol and 1,3,17β-triacetoxy-8α-estra-1,3,5(10)-triene] produced estrogenic effects in the mouse vagina, but not in the uterus. It may be that β-HCH also behaves in a selective manner, having qualitatively different effects in the vagina and uterus.

Concentration comparison to humans. The biological response to the low levels of these compounds was unexpected. Table 2 shows that the concentrations of OC pesticides in different unexposed human populations were generally only 2.7–120 (*o,p′*-DDT) or 2–140 (β-HCH) times lower than concentrations found to cause estrogenic responses in mice. However, in Israel, *o,p′*-DDT blood concentrations were found to be as high as 32 ng/mL (*28*), nearly double the minimal estrogenic blood level of 18 ng/mL observed in this study. Argentinean pesticide workers had blood β-HCH concentrations as high as 240 ng/mL (*29*); this is nearly six times the minimal estrogenic level of 42 ng/mL found in this study.

Although it is difficult to determine if the response in humans is the same as in mice, it is alarming that the human blood concentrations are so similar to the estrogenic concentrations in mice. The mouse has proven to be a good model for approximating estrogenicity of a compound in humans. For example, although the antiestrogens tamoxifen and clomiphene show little estrogenicity in the standard uterine weight and vaginal cornification bioassays performed in the rat, they are fully estrogenic in the mouse (*30–33*), and these compounds behave as estrogen agonists in the human uterus and vagina (*34–36*). Also, the mouse model of developmental effects of estrogens in the female and male reproductive tracts accurately reflects the effects that diethylstilbestrol had on developing human reproductive

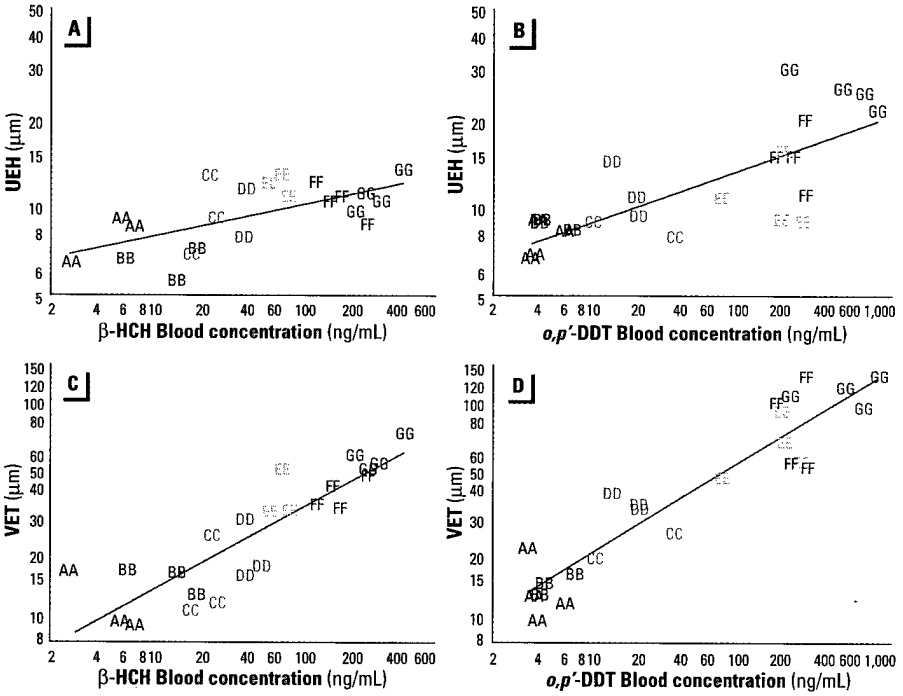

Figure 3. UEH and VET dose–response curves for β-HCH (*A* and *C*, respectively) and *o,p′*-DDT (*B* and *D*, respectively). Samples for which there were paired response and blood concentration values for β-HCH or *o,p′*-DDT were used in log–log plots. Individual samples from the different dosages (1/8, 1/4, 1/2 ... 4 capsules) are shown with letters AA, BB, CC ... GG, respectively, (compare treatment groups listed in Table 1). The square of the correlation coefficient, r^2, for UEH was 0.39 for β-HCH ($p < 0.002$) (*A*) and 0.58 for *o,p′*-DDT ($p < 0.0001$) (*B*). The r^2 for VET was 0.75 for β-HCH ($p < 0.0001$) (*C*) and 0.87 for *o,p′*-DDT ($p < 0.0001$) (*D*).

Table 2. Concentrations of β-HCH and *o,p′*-DDT reported in human blood.

Sample description	β-HCH (ng/mL)	*o,p′*-DDT (ng/mL)
Canada 1992[a] (*25*)	ND–2.6	ND–0.27
India male 1982 (*26*)	0.30 ± 0.03	0.15 ± 0.02
Pakistan 1987 (*28*)	0–7.2	
Yugoslavia 1985–1986 (*28*)	1.7	
Israel 1984–1985 (*28*)		ND–32
United States (NOE) 1971[a] (*29*)	1.4 ± 0.46	
Argentina (NOE) 1971[a] (*29*)	23 ± 10	0.27 ± 0.32
Argentina pesticide workers 1971[a] (*29*)	240 ± 140	
Japan 1986–1988 (*46*)	0.7–5.5	
England 1967 (*47*)	4.1	
Japanese in Florida (USA) 1971[a] (*48*)	14–17	1.5–2.2
Taiwanese in Florida (USA) 1971[a] (*48*)	3.6–21	Trace–6.6
Florida (USA) 1971[a] (*48*)	0.4 ± 2.0	0.7–2.0
India (NOE) 1992 (*49*)	13 ± 5.0	
India pesticide workers 1992 (*49*)	33 ± 23	1.5 ± 4.5
Mice with estrogenic response (this study)	42 ± 3.6	18 ± 2.3

Abbreviations: ND, not detected (or below detection limit of the assay used); NOE, nonoccupationally exposed.
[a]No sampling date was listed; therefore, the year of publication is given.

tracts (*37–39*), whereas the tests performed in a primate model were uninformative (*40*). On the other hand, the choice of the outbred CD-1 mouse may not have been optimum for determination of estrogen sensitivity; Spearow et al. (*41*) found that other strains are much more sensitive to the effects of estradiol in the developing testes. Similarly, we have found that strain differences can affect the outcome of the rat vaginal bioassay for xenoestrogen activity (*42*). Which strain of mouse is most appropriate for comparison to the human situation is not known, but certainly the results of the present study indicate that blood levels of *o,p'*-DDT or β-HCH in the nanograms per milliliter range have the potential of producing estrogenic effects.

This study was designed to produce blood and tissue levels of *o,p'*-DDT or β-HCH in mice comparable to those found in people from the general population, that is, those who are not exposed through a job-related or accidental event. Humans are exposed to OCs on a daily basis through respiration, foods, and contact (*16*). Additionally, circulating levels may increase during periods of weight reduction. As is generally seen in human studies, (*25, 26*), the concentration of OC pesticides is much higher in fat stores. Fat concentration is in equilibrium with blood concentration; thus, during periods of decreasing weight and fat stores, the compounds in fat are released into the blood stream and surrounding tissues (*43,44*). Bigsby et al. (*19*) showed that this OC release from fat can cause estrogenic responses in mice. This is especially important in fatty areas like the breast, in which the parenchyma is the target of estrogenic compounds that may act as tumor promoters (*16,45*).

This study is the first to measure tissue or blood levels of OC pesticide concentrations and correlate them with estrogenic responses in a laboratory animal. The extremely low levels required to cause statistically significant effects compared to control animals were unexpected. It is even more alarming that there is little difference between these levels and those that can be found in humans. Future studies that include long-term, low-dose exposure are required to simulate the chronic low-level exposure seen in humans.

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Understanding Enantioselective Processes: A Laboratory Rat Model for α-Hexachlorocyclohexane Accumulation

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Since cyclodextrin gas chromatography columns became popular for chiral separations, many researchers have noticed high enantiomeric ratios [ER: (+)-enantiomer/(−)-enantiomer] for α-HCH in the brains of wildlife. This investigation used the laboratory rat as a model for these phenomena. Rats were either pretreated with phenobarbital (PB) or left untreated and then dosed with α-HCH. Animals were sacrificed after 1 or 24 h. The ER averaged 0.95 ± 0.01 in blood, 1.29 ± 0.02 in fat, and 0.77 ± 0.004 in liver. ERs in brain ranged from 2.8 ± 0.5 to 13.5 ± 0.4. Both the tissue concentration distribution and the ERs agree well with those previously reported in wildlife. To determine whether high brain ERs were due to enantioselective metabolism or transport through the blood–brain barrier, α-HCH exposed brain and liver tissue slices were compared. Concentrations in the brain slices did not decrease with PB pretreatment but did decrease in the liver slices. Enantiomeric ratios in the brain slices averaged 1.11 ± 0.02 and were 0.76 ± 0.03 in liver slices for the PB pretreated rats. These data indicate that the enantioselective metabolism of α-HCH by the brain is not the mechanism responsible for high ERs in this tissue.

Introduction

Although organochlorine (OC) pesticides are very effective, they have been banned in most industrialized nations because of their persistence in the environment (*1*–*3*). One of the most widely detected OC pesticides is hexachlorocyclohexane (HCH) (*2*, *4*). The technical HCH mixture consists of eight isomers with the α-isomer existing as a pair of enantiomers. The γ-isomer, lindane, is the most effective pesticide component, but it only accounts for 10–12% of the technical HCH mixture. On the other hand, the α-isomer is not nearly as toxic to insects, but it accounts for 60–70% of the mixture. The main toxic action of HCH is central nervous system damage, and several studies have shown that these compounds accumulate in the brain of mammals where this toxic action occurs (*3*, *5*–*7*).

α-HCH is easily transported via the atmosphere, and it has contaminated all environmental matrixes, even those distant from sources (*2*). Concentrations of α-HCH in remote areas are about 60 pg/m^3 in air at an Arctic station at Alert, Canada (*1*, *4*), 3–34 ng/g dry weight in Arctic Ocean plankton (*8*), and 100–200 ng/g lipid in North Pacific and Arctic Ocean whale blubber (*9*). While α-HCH levels have been frequently studied, the processes that control their enantioselective environmental distribution had been largely neglected. However, once chiral gas chromatography (GC) columns became commercially available in the 1990s, the distribution of α-HCH enantiomers has been studied in water (*10*–*15*), snow (*16*), rain (*17*), soil (*18*–*20*), air (*11*, *12*, *17*, *21*), marine animals (*22*–*26*), and terrestrial animals (*13*, *25*, *27*, *28*). For example, α-HCH enantiomeric ratios [ER: amount (+)-enantiomer/amount (−)-enantiomer] in water have confirmed that marine bacteria degrade this compound enantioselectively (*14*), and Buser and Müller have studied the degradation of α-HCH in sewage sludge and determined unique rate constants for the loss of each enantiomer (*29*).

One of the most intriguing findings has been very high α-HCH enantiomeric ratios in the brains of neonatal fur seals (ER = 30) (*23*), sheep (ER = 1.4–3.7) (*28*), harbor seals (ER > 7.9) (*30*, *31*), and double-crested cormorants (ER > 3.6) (*25*). These ERs are among the highest measured in any matrix. There are three hypotheses to explain why the (+)-enantiomer of α-HCH is preferentially accumulated in the brain: (a) the blood–brain barrier selectively allows (+)-α-HCH into the brain, (b) the (−)-enantiomer is quickly metabolized in brain tissue, or (c) the (+)-enantiomer is selectively retained in the brain. The present study develops the laboratory rat as a model for these findings in wildlife. Phenobarbital was used to induce cytochrome P450, such as CYP2B1 and CYP2B2 (*32*, *33*), enzymes that are primarily responsible for the metabolism of xenobiotics (*34*). We also used rat tissue slices to determine if enantioselective metabolism is occurring in brain or liver tissues.

Experimental Section

Whole Rats. All procedures performed on animals followed the NIH *Guide for the Care and Use of Laboratory Animals* (*35*) and were approved by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine. Male Sprague–Dawley rats, 7–8 weeks in age, were divided into a control group or a phenobarbital (PB)-treated group. The PB rats were pretreated for 4 days with phenobarbital [75 mg/kg daily, intraperitoneal (i.p.)]; control rats were untreated. Twenty-four hours after the last injection of PB, all rats were injected with 25 mg/kg of α-HCH subcutaneously as a suspension in 40% ethanol in saline. The rats were killed by cervical dislocation at either 1 or 24 h after α-HCH injection. Each of the four groups contained six rats. Several rats were left untreated to provide tissues for blank and spike quality control samples.

Rat Tissue Slices. Four rats were used to further investigate the metabolism of α-HCH in their brain and liver. As before, two rats were pretreated with PB for 4 days (13 mg/kg daily, i.p.), and two rats were not treated. On the fifth day, all rats were sacrificed, and the liver and brain were removed. Each rat brain and liver was divided into two or three 2–3 mm thick slices of tissue. Two slices each of brain and liver from the control rats were incubated in 10.0 μg/mL of racemic

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α-HCH solution at 4 °C for reduced biological activity. The remaining 16 slices (8 control and 8 PB) were divided into two groups and incubated with either 1.0 or 10.0 μg/mL of racemic α-HCH. Incubation of these slices was conducted at 37 °C with shaking. All tissue slices were incubated for 6.5 h under 95% O_2/5% CO_2 in culture medium (DMEM-PRF/F12 with 0.05% bovine serum albumin, penicillin/streptomycin, glutamine, and HEPES buffer; from Sigma, St. Louis, MO). After incubation, all tissues were washed with culture media for 4−5 min at 4 °C to stop metabolism.

Liquid Extractions. Rat blood and the media involved in the tissue slice experiments were extracted with hexane. Sample volumes, ranging from 0.1 to 1.5 mL of liquid, were extracted. Unlabeled γ-HCH (Accustandard, New Haven, CT) was used as the internal standard, and 1 mL of 90% formic acid (Fisher Scientific, Pittsburgh, PA) was added. The resulting aqueous phase was extracted with 20 mL of hexane (EM Science, Gibbstown, NJ) three times with 1 min of vortex mixing and 1 min of centrifugation.

Tissue Extractions. Approximately 0.25 g of brain, liver, or fat tissues was ground with approximately 15 g of 10−60 mesh anhydrous sodium sulfate (Fisher Scientific). Unlabeled γ-HCH was spiked into the Soxhlet thimble containing the mixture. The samples were then Soxhlet extracted for 24 h with 300 mL of 50% acetone in hexane (EM Science).

Lipid Analysis and Removal. All tissues, excluding blood, contained a considerable amount of lipids, which was removed before GC analysis. Lipid analyses were performed in duplicate by a gravimetric method. The removal of lipids from the extracts was performed using a gel permeation chromatography column. The glass column (2.5 cm × 100 cm) was packed with porous styrene divinylbenzene copolymer beads (SX8 Bio-Beads, Bio-Rad Laboratories, Hercules, CA). The solvent was 60% cyclohexane in dichloromethane (EM Science) flowing at 10 mL/min through the column. The lipids were eluted in the first 20-min fraction, and the HCHs were eluted in the following 40-min fraction.

Silica Column Cleanup. Sample extracts were reduced to about 1 mL by rotary evaporation and exchanged into hexane if necessary. These extracts were then run through a 1 cm diameter silica column consisting of glass wool, 20 cm of silica (grade 923 Grace Davison, Baltimore, MD, 1% HPLC-grade water deactivated), and topped with 1 cm of sodium sulfate. Three fractions were collected; the eluents for each fraction were 75 mL of hexane, 75 mL of 50% hexane in dichloromethane, and 75 mL of dichloromethane with a 10-mL switching volume for the new solvent. The HCHs eluted in the second fraction. All three fractions were rotary evaporated and solvent exchanged into hexane if necessary. The second fraction was further reduced to about 50 μL by a gentle stream of nitrogen (Gas Tech, Hillside, IL) and quantitatively transferred into a GC autosampler vial.

Instrumental Parameters. The samples were analyzed on a Hewlett-Packard gas chromatographic mass spectrometer (GC 6890, MS 5973) in electron impact ionization mode. A Supelco BetaDex-120 column (Bellefonte, PA, 30 m × 250 μm i.d., 0.25 μm film thickness) was used for enantiomeric separation and quantitation. The temperature program for separation began at 50 °C for 1 min, ramped at 20 °C/min to 130 °C, ramped at 1 °C/min to 170 °C, ramped at 30 °C/min to 230 °C, and held at 230 °C for 1 min for a total analysis time of 48.3 min. An autosampler made injections of 1 μL into a 230 °C injector in the splitless mode with a helium pressure of 24 psi. The transfer line into the mass spectrometer was held at 230 °C, the ion source was held at 230 °C, and the quadrupoles were held at 150 °C. The HCH compounds were monitored with selected ion monitoring (SIM) of m/z 181, 183, 185 $[M - Cl - 2HCl]^+$, 217, and 219 $[M - Cl - HCl]^+$. The expected relative abundances of these ions are 100, 98, 31, 70, and 90, respectively.

Quantitation. Each α-HCH enantiomer was quantitated by the relative response factor method. The m/z 181 ion was used for quantitation purposes, and the m/z 183 ion served to confirm the identity of the compound. The ratio of m/z 181:183 ions was required to be within 20% of standard values (100:98) to confirm the presence of α-HCH. This criterion is somewhat lenient because of the differences in the mass spectra and because there should only be one easily detected OC pesticide in the rats.

Quality Control. With each set of six samples, a matrix spike and either a matrix blank or a glassware blank were also extracted. All quality control samples underwent the same cleanup procedure as the samples. The matrix spike contained a known amount of the target analyte at a concentration similar to what was expected in the samples. Spike recoveries ranged from 72 to 132% with an average and standard deviation of 85 ± 16%; sample concentrations were not corrected for recovery. No target compound was found in any type of blank; therefore, blank corrections were not necessary.

Peak Fitting. Peak fitting was necessary to accurately determine the enantiomeric ratio for α-HCH in biological samples, which had overlapping peaks making integration difficult. As previously described, Peak Fit version 4.0 (Jandel Scientific, San Rafael, CA) was used for this task (*36*). Peak fitting was performed separately for the five SIM ions recorded in the mass spectral analysis. Peak areas, standard errors, r^2 values, peak centers, and resolution results were then copied to spreadsheets. Quality control measures were used on these data to ensure accurate measurements. The maximum difference between the ER for the five ions was required to be ≤10% of the average ER for that sample. If one ion was significantly different from the other four, that ion was removed and the condition applied again. This criterion usually failed due to a low signal of the ion at m/z 185. Also, the isotopic area ratios of the ions at m/z 181:183 for each of the two enantiomers were required to be within 10% of each other. The isotope ratios of samples were required to be within 10% of the average of the standards (100:98). All ERs meeting these requirements were considered reliable and were normalized by dividing by the average ER of the racemic standards for a given set of samples. This removes any bias that the column or instrument might introduce.

Statistical Analysis. Enantiomeric ratios were compared to the racemic value of 1.00 by a two-tailed t-test ($\alpha = 0.05$) using the TDIST function in Microsoft Excel. The enantiomeric ratios for each treatment in a given tissue were compared to each other by a one-way ANOVA ($\alpha = 0.05$, two-tails) using SigmaStat 2.03 (SPSS Inc., Chicago, IL). If significant differences were found, multiple comparisons were made by a Tukey test. A one-tailed t-test was used to determine if PB-pretreated animals had lower concentrations than control animals ($\alpha = 0.05$).

Results and Discussion

Concentrations. Figure 1 shows several interesting trends in the average concentrations (note the logarithmic scale) for all groups of treated rats in this study. Not surprisingly, given the lipophilicity of this compound, the highest α-HCH concentrations are found in the fat after 24 h, and the lowest concentrations are found in the blood. The average and standard error of the percent lipid in the fat, brain, and liver are 78 ± 1.8, 13 ± 1.5, and 10 ± 1.5, respectively. In the 1-h animals (control and PB), the α-HCH concentration in the brain is on the same order of magnitude as the fat. This indicates that brain tissue is capable of accumulating a large amount of α-HCH before sufficient time has passed for metabolism.

In this experiment, phenobarbital was used to induce the P450 enzymes that metabolize compounds such as α-HCH

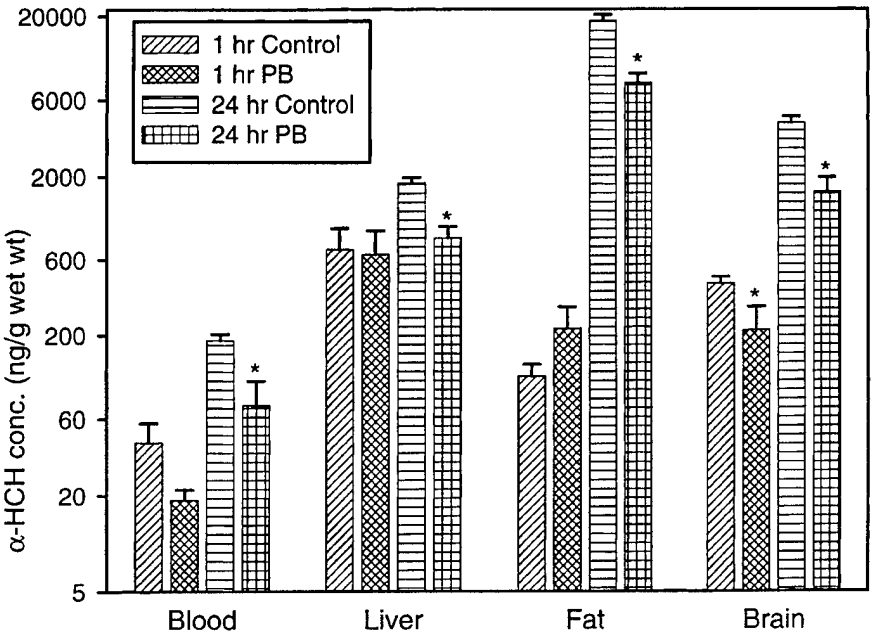

FIGURE 1. Logarithms of the α-HCH concentrations (ng/g wet weight) for all tissues (blood, liver, fat, and brain) and treatment groups (1 h control, 1 h phenobarbital, 24 h control, and 24 h phenobarbital). Error bars are standard errors; $n = 4$. Phenobarbital bars marked with an asterisk (*) have a statistically lower concentrations than the control animals at the same time period ($p < 0.05$).

TABLE 1. α-HCH Distribution in Rats[a]

	blood	brain	liver	fat	total
1 h control	415 ± 270 (0.011)	455 ± 89 (0.012)	5 630 ± 4 100 (0.15)	2950 ± 1 100 (0.079)	9450 ± 4 200 (0.25)
1 h PB	182 ± 64 (0.0049)	228 ± 190 (0.0061)	5 210 ± 4 300 (0.14)	5900 ± 4 300 (0.16)	11 500 ± 6 100 (0.31)
24 h control	1 800 ± 360 (0.048)	4 650 ± 430 (0.12)	14 800 ± 2 400 (0.39)	502 000 ± 96 000 (13)	523 000 ± 96 000 (14)
24 h PB	707 ± 590 (0.019)	1 730 ± 810 (0.046)	6 620 ± 2 400 (0.18)	206 000 ± 56 000 (5.5)	215 000 ± 56 000 (5.7)

[a] Values are the total mass (ng) ± standard deviation of α-HCH in each tissue or summed for the four tissues. Numbers in parentheses are the percent of α-HCH expressed as the percent of the total administered dose of 3.75×10^6 ng.

in the rat. Specific enzymes were not of concern, but CYP2B1 and CYP2B2 are known to be induced by this treatment (*32, 33*). As expected, the average α-HCH concentration of a given tissue at 24 h is statistically lower in the phenobarbital treated animals as compared to the control animals, confirming that P450 enzymes that metabolize α-HCH have been induced. Even at 1 h, PB caused a significant decrease in the concentrations in brain samples; this may be the result of the lower α-HCH concentration in the blood for this treatment, making less α-HCH available for uptake in the organs.

Table 1 presents distribution data in the four measured tissues and in the four treatment groups. The concentration in each tissue was averaged (see Figure 1) and multiplied by a known or estimated organ weight for a 150-g male Sprague–Dawley rat (blood, 9.75 g; brain, 1.08 g; liver, 8.1 g; ref *37*; fat, 27 g estimated). This calculation gives the mass of α-HCH in each tissue in nanograms. The mass in the four tissues was then summed, and this is presented in the far right column of Table 1. The α-HCH mass in each tissue is also expressed as a percent of the injected dose (3.75×10^6 ng).

The entire α-HCH dose in this study was given by subcutaneous injection. At 1 h, we have only accounted for 0.25% (control) and 0.31% (PB) of the total dose in the four tissues measured. It is likely that after 1 h there is still a large pool of α-HCH at the injection site. After injection, the α-HCH is carried to various organs via the blood stream. Notice that the PB-pretreated animals have a lower amount in the blood than the control animals. The α-HCH arrives at the liver, where the P450 enzymes start a series of reactions to metabolize these compounds. Notice that there is a slightly lower mass in the liver of PB animals than in controls, presumably due to the induction of metabolizing enzymes. Even at 1 h, there is also a significant portion of the α-HCH that accumulates in the brain (455 ng of control and 228 ng of PB animals). The mass of α-HCH in the fat varies depending on the treatment, with a higher percentage stored in fat for the PB-pretreated animals (0.079% in control versus 0.16% in PB-pretreated rats). There is a shift in concentration from blood, brain, and liver in control animals to fat in 1-h PB-pretreated animals.

After 24 h, more α-HCH has moved away from the injection site, and we have accounted for a larger portion of the total α-HCH dose in the four tissues measured (14% control and 5.7% PB). Much of the injected α-HCH has probably been taken up into the blood stream and distributed to the organs or excreted. Incredibly, nearly all of the measured α-HCH has accumulated in the fat (up to 13% of the total dose). The fraction of α-HCH in the blood after 24 h has increased from 0.011% to 0.048% in control animals and from 0.005% to 0.019% in PB-pretreated animals. A

TABLE 2. Ratio of Tissue Concentrations of HCH in Different Organs[a]

	fat:blood	fat:liver	fat:brain	brain:liver	ref
Finnish humans (γ-HCH)	0.6–60				(*39*)
Canadian humans (β-HCH)	43–355				(*40*)
Delhi, India humans (α, β, γ-HCH)	12–47				(*41*)
Lucknow, India humans (γ-HCH)	65				(*42*)
harbor seal (γ-HCH)		45–60			(*43*)
beluga whale				24	(*44*)
α-HCH dosed rats	27–250	1.8–16	2.1–48	0.16–0.85	(*45, 46*)
double-crested cormorant				2.3	(*25*)
neonatal seal		0.7	0.6	1.2	(*23*)
stillborn seal		1.4	0.5	3.0	(*23*)
diseased neonatal seal		2.7			(*23*)
rats 1 h control	2.6	0.2	0.3	0.6	this work
rats 1 h PB	12	0.3	1.0	0.3	this work
rats 24 h control	100	10	4.3	2.4	this work
rats 24 h PB	110	9.3	4.8	2.0	this work

[a] Unless noted, ratios are for α-HCH.

similar trend is noted in the liver, and also notice that the percent decreases for all tissues in the PB animals as compared to control animals. It is also remarkable that such a large percentage of the dose has accumulated in the brain; control and PB animals have 0.12% and 0.046%, respectively. This observation has important implications for potential neurological effects.

One of the goals of this work was to determine if the laboratory rat could be a useful experimental model for determining the mechanisms that account for the observations in wildlife. For comparison, Table 2 lists tissue concentration ratios from the literature and those determined in this study. All tissue-to-tissue ratios are for α-HCH, except where noted, and are on a wet weight basis. The values for the fat-to-blood comparison range between 0.6 and 355. The fat-to-blood ratios in our rats ranged from 2.6 to 110. Such high fat-to-blood ratios are another indication of the high lipophilicity for α-HCH. The fat-to-liver ratio ranges from 0.7 in neonatal seals to 60 in harbor seals. The rats in this study had fat-to-liver ratios of 0.2–10, falling slightly below to within the reported range in wildlife. The fat-to-brain ratio ranges from 0.5 in stillborn seal to 48 in fed rats, while the rats in this study ranged from 0.3 to 4.8. The rat fat-to-brain ratios in this study fall mostly within this range. The brain-to-liver ratio in wildlife ranges from 0.16 in fed rats to 24 in Beluga whales. The ratio in the rats of this study lies on the lower end ranging from 0.3 to 2.4. Overall, the distributions of α-HCH in rats from this study matches reasonably well with the distributions reported in wildlife, making the rat an acceptable model for this study.

Enantiomeric Ratios. The enantiomeric ratios of α-HCH in each tissue and for each treatment are shown in Figure 2. The dotted line shows a racemic ratio of 1.00. Out of the 16 possible combinations of treatments and tissues, all except four were found to be statistically different than racemic at $p < 0.05$. One of these racemic categories is the 1-h PB-pretreated rat fat. This value does not appear to have a racemic average, but the large error associated with the measurement makes it statistically impossible to distinguish it from racemic. Three of the four blood ERs cannot be distinguished from racemic, and the fourth has an average reasonably close to 1.00 (1-h control ER = 0.93 ± 0.02). The preferential accumulation of one enantiomer in tissues suggests that there are enantioselective processes occurring in the rat. However, it is not possible to determine from the ER alone whether the nonracemic values are due to enantioselective differences in uptake, metabolism, or removal from these tissues.

Blood ERs are close to 1.00, but are generally slightly lower, indicating a small preferential storage of the (−)-enantiomer or loss of the (+)-enantiomer. There is no detectable statistical difference in α-HCH blood ERs between rats with different treatments (one-way ANOVA, $p > 0.05$). Upon searching the literature, we were unable to find any reported α-HCH blood ERs from wildlife.

In comparison, liver α-HCH ERs are also less than 1.00 but to a greater extent than in the blood (averages for all treatments in liver 0.77 ± 0.004 and blood 0.95 ± 0.004). Again, there was no statistical difference in α-HCH liver ERs between rats with different treatments. There are some species such as the common eider duck (*13*), cormorants (*25*), and fur seals (*23*) that have liver α-HCH ER values greater than 1.00. However, the rat ERs agree with many findings in wildlife where liver ERs were 0.76–0.98 in flounder (*13*), 0.03–0.40 in roe deer (*27*), and 0.68–0.98 in sheep (*28*). Thus, as in several wildlife species, there is a preferential accumulation of the (−)-enantiomer or loss of the (+)-enantiomer in the rat liver.

On the other hand, the enantiomeric ratio in fat (overall average = 1.29 ± 0.02) is greater than 1.00, indicating a preferential accumulation of the (+)-enantiomer or loss of the (−)-enantiomer. Again, there is no statistical difference in α-HCH fat ERs between rats with different treatments. Given that up to 13% of the total α-HCH dose is stored in the fat, this abundance of the (+)-enantiomer may lead to a corresponding excess of the (−)-enantiomer in other tissues such as the liver and blood. These data are consistent with most other reported fat and blubber ERs. Values are 0.10–0.97 in Antarctic weddell seals (*38*), 0.19–0.75 in hooded seals (*24*), 1.14–1.57 in Icelandic seals (*22, 38*), 1.02–2.45 in Baltic and North Sea seals (*24*), 1.56–1.58 in Japanese northern fur seals (*25*), and 1.6–2.8 in various marine species from the North Pacific and Bay of Bengal (*26*).

In the brain, there is no statistical difference between the α-HCH ERs for the 1-h treatments, but the other treatments are statistically different from each other (one-way ANOVA, $p < 0.05$). The average ER changes dramatically from the 1- to 24-h samples (2.77 ± 0.35 at 1 h, 10.7 ± 1.3 at 24 h). After 24 h, the PB animals have a significantly more nonracemic ER than the control animals. These α-HCH brain ERs agree well with those previously reported in the literature for neonatal fur seals (ER = 30) (*23*), harbor seals (ER > 7.9) (*30, 31*), sheep (ER = 1.36–3.72) (*28*), and double-crested cormorants (ER > 3.6) (*25*). Thus, the laboratory rat is a good model for α-HCH enantiomer accumulation in many species of wildlife. Whole rat studies indicate that all tissues, except

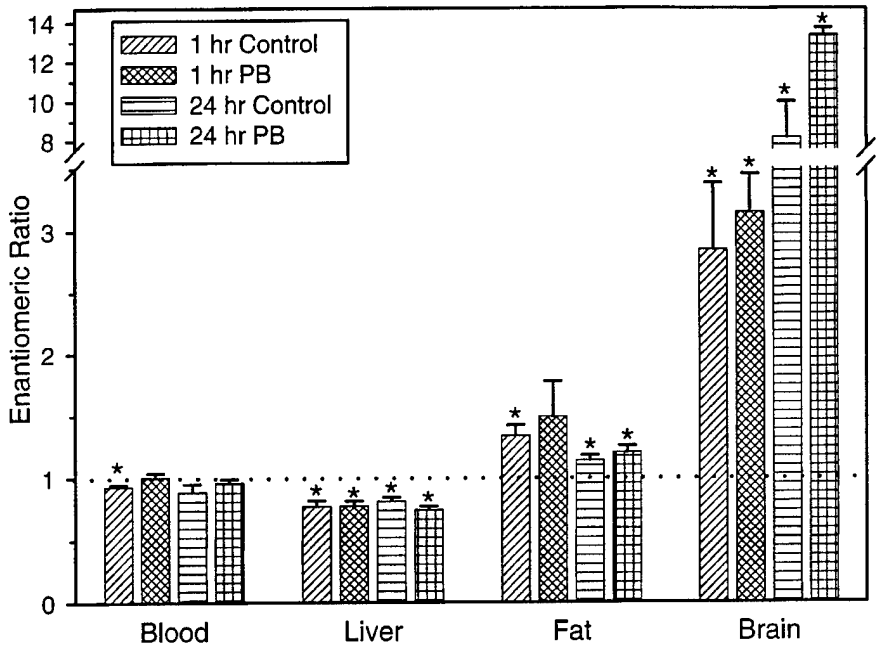

FIGURE 2. Enantiomeric ratios [ER: amount of (+)-enantiomer divided by amount of (−)-enantiomer] of α-HCH for all tissue and treatment groups in whole rats. Dotted line at 1.00 indicates a racemic mixture. No statistical differences were detected within tissues for different treatment groups in blood, liver, and fat (one-way ANOVA, $p > 0.05$). Error bars are standard errors; $n = 4$. Y-axis has 3.5−7.5 omitted. Bars marked with an asterisk (*) are statistically different than an ER of 1.00 ($p < 0.05$).

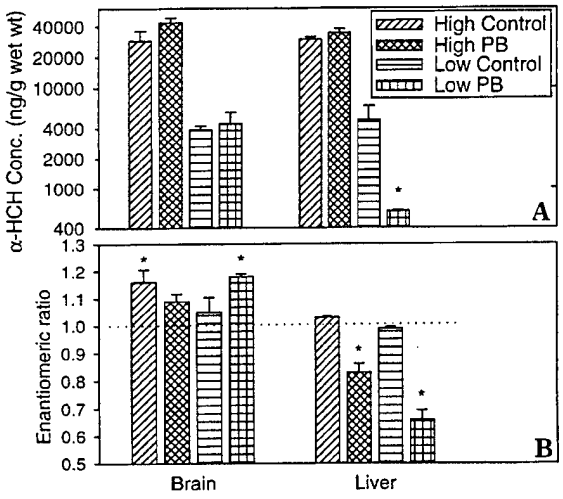

FIGURE 3. Rat tissue slice incubation data. (A) Logarithms of the α-HCH concentrations (ng/g wet weight) in brain and liver tissue slices. Bars marked with an asterisk (*) are statistically different than the controls ($p < 0.05$). (B) Enantiomeric ratios of α-HCH in brain and liver tissue slices. Dotted line at 1.00 indicates a racemic mixture. Bars marked with an asterisk (*) are statistically different than 1.00 ($p < 0.05$). All errors bars are standard errors.

blood, have enantioselective processes that influence uptake, metabolism, and elimination.

Rat Tissue Slices. One hypothesis to explain the high ERs in brain is the relatively rapid metabolism of (−)-α-HCH in the brain. To test this theory, we devised another experiment using rat tissue slices. By using in vitro slices, the possibility that the blood−brain barrier caused enantioselectivity is removed from the system. Figure 3 shows both concentration and enantiomeric ratio data for these experiments. Figure 3A gives the α-HCH concentrations in the tissue slices. The tissue slices were incubated in two different doses of α-HCH (10 and 1.0 μg/mL), and it is apparent from the figure that the tissue concentrations reflect this difference.

In the higher dose tissue slices, there was no statistical difference between α-HCH concentrations in the control and PB-pretreated rat brain and liver tissue slices. In this case, the higher dose may have been overwhelming to the tissues, and 6.5 h may not have been enough time to cause any detectable change in concentration in either tissue. However, the low dose liver slices did show a drastic decrease between the control tissue slice and the PB pretreatment (control slice = 4800 ng/g versus PB slice = 590 ng/g). It is important to note that, unlike the whole rats, the brain tissue slices do not show any sign of PB-induced metabolism; that is, the concentrations in the slices from control and PB-treated animals are similar in the in vitro study. However, in the whole rats, there was a decreased concentration in brains from PB-treated rats following α-HCH injection, perhaps due to liver metabolism decreasing the amount of α-HCH available.

Figure 3B shows the α-HCH enantiomeric ratio data for the tissue slices. In liver tissue slices, the control and low temperature (not shown) tissue slices have ER values that are indistinguishable from racemic, but the PB-pretreated tissues have an average α-HCH ER of 0.76 ± 0.03. This tissue slice ER is in good agreement with the liver ER in whole rats (0.77 ± 0.004). The combination of decreasing concentration by a factor of 8 and the shift to nonracemic ERs in PB-pretreated animals verifies that the liver tissue slices are selectively metabolizing (+)-α-HCH.

The ER in the brain tissue slices are greater than 1.00 just as in the whole rats. However, the overall average of ER in the brain slices (1.11 ± 0.02) is barely nonracemic, is similar to the ERs for tissues incubated at 4 °C (not shown, ER = 1.05 ± 0.01), and is nowhere near the values in whole rats of 2.77 ± 0.35 at 1 h. Since the tissue slices incubated at low and high temperatures had similar ERs, it appears that the enantioselectivity in brain tissue slices is due to selective uptake of the (+)-enantiomer. Similar α-HCH concentrations in control and PB brain tissue slices combined with near

racemic ERs indicate that metabolism cannot be responsible for the large brain ERs detected in both wildlife and the laboratory rat model. Other mechanisms, such as selective passage of the (+)-enantiomer through the blood–brain barrier or selective retention of (+)-α-HCH must be the cause of the very high enantiomeric ratios of α-HCH in the brain. Further studies using single enantiomers of α-HCH are required to distinguish between these possibilities.

These experiments have demonstrated that the laboratory rat is a good model for the α-HCH findings in wildlife and can be used for future investigations. We have also shown that preferential metabolism of the (−)-enantiomer of α-HCH in brain tissue is not contributing to the high enantiomeric ratio in this organ. These results agree with several other researchers, who have suggested that the blood–brain barrier was responsible for the high ER in brain tissue (*23*, *28*, *30*, *31*).

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Synergistic Tumor Promoter Effects of Estrogens and Progesterone in Methylnitrosourea-induced Rat Mammary Cancer

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ABSTRACT

Estrogen and progesterone are believed to act as tumor promoters in the mammary gland; however, this has not been tested in a classical tumor-initiator, tumor-promoter animal model. Rats were ovariectomized, treated with methylnitrosourea (MNU) and then with estradiol (E_2), estrone (E_1), and/or progesterone (P_4) by subcutaneous implantation of a slow release capsule. Tumor incidence was 0-3% in ovariectomized rats and 98-100% in ovary-intact animals. Treatment of ovariectomized animals with E_2 increased tumor incidence to 24% ($P = 0.053$, vs. ovariectomized control) while E_1 treatment yielded a 54% tumor incidence ($P < 0.0001$, vs. ovariectomized control). P_4 administered alone did not have any tumor promoter effect, but when P_4 and E_1 were administered together tumor incidence was 88% ($P = 0.042$ vs. E_1 alone). Whole mounts of mammary glands from ovariectomized rats showed that treatment with either E_1 or E_2 induced moderate growth. In contrast, the combined treatment of P_4 plus E_1 had a synergistic effect on mammary gland growth. On the other hand, it is known that administration of estrogen to ovary-intact animals prevents MNU-induced tumorigenesis. In this study, treatment of intact animals with E_2 produced a greater reduction in mammary tumor incidence than did E_1. Tumor incidence was 56% and 19% in E_1- and E_2-treated intact animals, respectively (relative risk for E_2-treated vs. E_1-treated: 0.47, CI: 0.236-0.922). Both estrogens induced a similar extent of glandular development in these animals. These observations suggest that the tumor promoter activity of a particular estrogen depends upon a balance between its tumor promotive and its anti-tumor actions. Furthermore, P_4 synergizes with E_1 to promote tumorigenesis, supporting the notion that combined estrogen plus progestin hormone replacement therapy may pose an increased risk in postmenopausal women.

INTRODUCTION

Several lines of evidence point to estrogens as playing a prominent role in mammary gland tumorigenesis. Estrogens induce growth of the glands as demonstrated in animal models (1, 2) and in humans (3-5). Epidemiological data strongly indicate that breast cancer incidence is related to a woman's total lifetime exposure to ovarian hormones and estrogen is believed to be the essential hormone in this phenomenon (6-9). These inferences are supported by rat models of carcinogen-induced mammary tumors showing that growth of the tumors is dependent upon estrogen, i.e. when animals bearing tumors are ovariectomized the tumors regress if no exogenous estrogen is supplied (10-12). Together, these observations suggest that estrogens should be considered important tumor promoters in the breast.

On the other hand, estrogens can be used to diminish tumor growth in women or prevent tumorigenesis in carcinogen-treated animals. Over 50 years ago it was recognized that administration of large doses of estrogen would cause tumor regression in approximately 30% of breast cancer patients (13, 14). When estrogen is administered to rats prior to, or within a week of treatment with the potent carcinogen, methylnitrososurea (MNU), the incidence of mammary tumors is dramatically reduced (15-17). Estrogen's protective effects are believed to stem from its capacity to induce cellular differentiation, thereby blocking further growth (18).

Until recently, a role for P_4 in mammary tumorigenesis has been less clear than for estrogen. Although there were indirect indications that P_4 increased proliferation of mammary epithelial cells (6), experimental results with human breast cancer cell lines have been conflicting (19-22). Although medroxyprogesterone acetate induced mammary

tumors in beagle dogs, this was considered anomalous, as there was no indication that it did so in humans (23, 24). Nor did epidemiological studies show a clear influence of postmenopausal progestin treatment (25, 26). However, more recent data indicates that postmenopausal women receiving a hormonal regimen of combined estrogen plus progestin are at greater risk of developing breast cancer than those receiving only estrogen (27, 28). Animal studies support the notion that progestins play a role in growth of mammary epithelium (29-31) and studies with progesterone receptor knockout mice indicate that P_4 is essential for carcinogen-induced mammary tumors (32).

In the mammary gland, steroid hormones are thought of as tumor promoters, compounds that stimulate proliferation of cells that have been subjected to an initiation event, but this has not been adequately tested. The rat model of MNU-induced mammary tumors presents us with an ideal system in which the tumor promoter character of a hormone can be examined. MNU is a powerful alkylating agent that is completely oxidized and removed from the animal's circulation within minutes of its administration (33-35). A single injection of MNU will induce mammary tumors in 90-100% of mature, ovary-intact rats within 6 months (reviewed in ref. 6). When tumor-bearing rats are ovariectomized, the MNU-induced tumors regress and administration of estrogen stimulates regrowth of the tumor (6). These observations have been interpreted as evidence that estrogens behave as tumor promoters in the mammary gland. However, such experiments miss the opportunity to actually test the tumor promoter activity of hormones in a classic initiator-promoter analysis because tumor promotion has already occurred before ovariectomy was performed. The proper experimental design would test the ability of estrogen to promote tumorigenesis in ovariectomized animals after receiving the initiator, MNU. In a slightly unconventional experiment, Kumar et al (36) showed that E_1 promoted mammary tumors in 50% of rats that had been treated with MNU neonatally, maintained on tamoxifen, ovariectomized as juveniles, and then treated with hormone. There are no other reports in which ovarian

hormones have been tested for their tumor promoter activity in ovariectomized animals undergoing chemically-induced tumor initiation.

In the present study, we examined the tumor promoter activity of E_2, E_1, and P_4 in ovariectomized, Sprague-Dawley rats. We found that neither estrogen alone would provide full promoter activity. Furthermore, it appears that E_2 and E_1 exhibit reciprocal degrees of tumor protective and tumor promoter activity, that is, compared to E_1, E_2 was better at inhibiting tumorigenesis in intact rats but it was a weaker tumor promoter in ovariectomized rats. A combination of E_1 and P_4 produced full tumor promoter activity in ovariectomized rats. Such observations support the notion that combined hormone therapy may lead to increased risk for breast cancer compared to estrogen alone.

MATERIALS & METHODS

Experimental treatment of animals was performed under protocols designed according to guidelines provided by *The Guide for the Care and Use of Laboratory Animals* (37) and approved by the Institutional Animal Care and Use Committee. To test the tumor promoter activity of hormones, Sprague-Dawley rats (49-56 days old) were ovariectomized and treated with 50 mg/kg MNU (Sigma Chemical Co., St. Louis, MO) by tail vein injection while still under anesthesia (ketamine). At the time of ovariectomy, a treatment capsule was implanted subcutaneously in each animal. The capsules were made from Silastic tubing (Konigsberg Instruments, Pasadena, CA, 0.062 in. I.D. X 0.125 in. O.D. X 1.4 cm length); each capsule contained 26-30 mg of crystalline steroid and was sealed at each end with Silastic cement. The tumor promoter effects of E_2, E_1, and P_4 were tested separately by implanting a single capsule; groups of animals were also treated with P_4 in combination with E_1 by implanting two capsules, one of each steroid. As a positive control, ovary-intact

rats were implanted with empty Silastic capsules and treated with MNU. As negative controls, ovariectomized, MNU-treated animals were implanted with an empty capsule. In addition, the protective effect of estrogens delivered by the Silastic implant method was tested in two groups of animals that were ovary-intact, treated with MNU and had a single capsule of E_2 or E_1 implanted at the time of MNU treatment. Beginning at 4 weeks after treatment and continuing through 8 months, animals were palpated on a weekly basis to determine presence of mammary tumors.

It was of interest to determine the mammary tissue growth responses to the various hormone treatments early in the experimental protocol. Accordingly, ovariectomized or intact animals were treated with hormone for 1 month and killed for collection of mammary glands for whole mount observation (see below).

At the end of the experiment, animals were killed by cervical dislocation. Whole body, pituitary, and uterine weights were recorded; tumors were collected and processed for histological examination. Each animal was skinned to allow visualization of the mammary glands and to determine if small tumors had escaped detection during palpation.

Mammary glands were also examined by the whole mount technique. The skins were removed from each animal, with mammary fat pads intact. The skins were immersed in Tellyesnickzky's fixative (0.05% glacial acetic, 1.85% formaldehyde, 70% ethanol) overnight. The mammary fat pads were removed and immersed in graded ethanol solutions (70%, 95%, 100%) for 45 min. each and then defatted by immersion in an acetone bath with 2 changes of 1 h duration and then overnight in fresh acetone. The tissue was then rehydrated and stained in 0.1% toluidine blue for 2 h. The fat pads were destained in methanol and 70% ethanol for 30 min. each and washed in water before immersion in 4% ammonium molybdate for 30 min. to fix the stain. The fat pads were then cleared by

dehydration through graded alcohols and xylene. The lightly stained glands were viewed under a dissecting microscope that was interfaced with a Macintosh PowerPC computer (Cupertino, CA) through a Sony CCD video camera. Using lymph nodes as landmarks, a standard region of an inguinal or a thoracic gland was imaged.

Tumor incidence was analyzed by Fisher's exact test. All other parameters were analyzed by ANOVA, followed by Fisher's LSD test for differences between individual treatment groups.

RESULTS

In an initial experiment, the appearance of tumors was monitored in ovariectomized rats receiving either E_2 or E_1. Tumor incidence reached 100% in ovary-intact animals within 5 months of MNU treatment; none of the ovariectomized animals developed tumors (Figure 1). E_2 treatment resulted in only a 24% tumor incidence while E_1 produced tumors in 55% of animals (Figure 1 and Table I). Tumor incidence in E_2-treated animals did not differ statistically from ovariectomized controls while that of the E_1-treated group differed from the ovariectomized controls ($P < 0.05$) but, in this experiment, not from intact controls (Table I). In contrast, E_2 had a greater effect than E_1 on body weight and pituitary weight (Table I). Tumor latency in both E_2 and E_1 treated groups did not differ from that of intact controls.

The above results demonstrated that although E_1 proved to be a better tumor promoter than E_2, its was not fully effective compared to intact controls, suggesting that the ovary supplies a factor in addition to estrogen for tumor promotion. Therefore, further experiments were performed to test the effect of E_1 in conjunction with P_4 (Figure 2). Also, data from the first

experiment (above) were combined with the new experimental results to further examine the difference between E_2 and E_1 tumor promoter efficacy. Analysis showed that there was a tendency for an increased tumor incidence under E_2 stimulation ($P = 0.053$ vs. ovariectomized control). E_1 treatment produced a tumor incidence that statistically differed from the ovariectomized control incidence ($P < 0.0001$) but was less than that of intact animals ($P < 0.001$). Also, the difference in tumor incidence between the E_1 and E_2 treatment groups tended to be different ($P = 0.072$). P_4 alone had no tumor promoter effect but it synergized with E_1 to produce a significantly greater tumor incidence than E_1 alone ($P = 0.042$). The tumor incidence in the P_4 plus E_1 group (88%) was not different from the intact control group (98%).

Because estrogen can inhibit tumorigenesis in the MNU-treated rat (15-17), the effect of E_2 and E_1 was also examined in ovary-intact animals. Both steroids had a protective effect, significantly reducing the tumor incidence (Figure 2). E_2 produced a greater degree of protection. Tumor incidence was 56% and 19% in E_1- and E_2-treated intact animals, respectively (relative risk for E_2-treated vs. E_1-treated: 0.47, CI: 0.236 - 0.922). Furthermore, the tumor incidence in the E_2-treated intact animals did not differ from ovariectomized controls.

Examination of the mammary glands of the ovariectomized, hormone-treated animals showed that estrogens stimulated growth. Glands of ovariectomized animals were characterized by terminal duct structures with no end bud formation (Figure 3 A & B). E_1 increased the number of lateral buds and end buds (Figure 3C & D). There was no apparent difference between the E_2 (not shown) or E_1 groups that would account for the higher incidence of tumors under E_1 stimulation. There was an additional increase in glandular structures in the P_4 plus E_1 group; in particular, there was a multiplication of terminal end buds and the appearance of alveolar buds (Figure 3E& F). Administration of

P_4 alone had no effect (not shown). In intact animals, both estrogens induced similar degrees of glandular development, increasing the number of lateral buds, terminal end buds and alveolar buds (Figure 4).

DISCUSSION

Although it is generally held that estrogen is the ovarian factor that most influences mammary tumorigenesis (6-9) there is recent evidence that exogenously administered progestin may play a part in development of breast cancer in postmenopausal women (27, 28). On the other hand, high doses of estrogens are known to inhibit human mammary tumor growth (13, 14). The MNU rat model offers a suitable system for testing the tumor promoter or anti-tumor action of hormones. Our results show that E_1 is more effective as a tumor promoter than E_2 in this model but that neither hormone was sufficient for full tumorigenesis. Kumar et al (36) also found that E_1 induced a tumor incidence of 50% in MNU-treated rats; however, the lack of a full tumorigenic effect of the estrogen in this case could be attributed to the use of an experimental protocol that deviated considerably from the standard model. It has been suggested that estrogen stimulated growth of MNU-induced tumors is an indirect effect through increased prolactin secretion from the pituitary (18, 38, 39). However, the present observation that E_1 is a better tumor promoter than E_2 does not support this notion because both hormones are likely to stimulate the pituitary to the same or greater extent; the size of the pituitary glands at the end of the experiment indicates that this is indeed the case. On the other hand, combined P_4 plus E_1 treatment induced tumor incidence comparable to that seen in ovary-intact animals. It is known that prolactin and P_4 interact to induce growth and development of mammary gland (29). In particular, progesterone receptor is involved in lobuloalveolar development (31). Lydon et al

have found that progesterone receptor was essential for carcinogen induced mammary tumors in mice under prolactin stimulation (32). Together with our observations, these reports indicate that P_4 is very important to the tumorigenic processes of the mammary gland.

It is likely that cellular proliferation plays a role in the tumor promoter activity of hormones. The appearance of mammary glands in ovariectomized rats treated with either estrogen alone indicates that the hormone stimulated a dramatic increase in glandular structures; glandular development was even more pronounced with estrogen plus P_4 treatment. We also found that P_4 administered alone was without effect. Thus, there is a synergistic induction of glandular growth under P_4 plus E_1; the increased epithelial proliferation required for this development probably contributes to the increased tumorigenesis in this treatment group. However, the difference in tumor incidence between the E_2 and E_1 groups cannot be readily explained on this basis because there was no apparent difference in the degree of glandular development between these two groups.

Estrogen, administered alone or in combination with P_4, prevents mammary tumor formation in carcinogen treated, ovary-intact rats (15-17). Using Silastic capsule implants identical to those used in the present study, Guzman et al (40) found that administration of E_2 plus P_4 for three weeks, beginning at two weeks after the MNU injection, reduced tumor incidence by greater than 80% in ovary-intact animals. There was a 62% reduction in tumor incidence when E_2 was administered alone; by itself, P_4 had no effect. Protection was also provided when hormones were administered for only one week. Similarly, in the present study E_2 reduced tumor incidence in intact animals by approximately 80% but E_1 reduced incidence by only 45%. Whole mount examination of mammary glands indicates that both E_2 and E_1 stimulated growth and differentiation of the mammary epithelium of intact rats. Although others had suggested that the reduction in tumor incidence was the result of hormone-

induced proliferation and differentiation of the glandular epithelium (16-18), Guzman et al found that perphenazine, a potent differentiating agent in the mammary gland, did not prevent tumorigenesis (40). Likewise, prolactin secretion from pituitary isografts induced fully differentiated glands in mice, yet these animals were highly susceptible to MNU-induced mammary tumorigenesis (41). Conversely, Sivaraman et al (42) showed that full lobuloalveolar differentiation was not necessary for the protective effects of E_2 plus P_4. Thus, the mechanism behind the protective effect of E_2 may prove subtler than gross differentiation of the glandular cells.

It is worth noting that the degree to which the particular estrogen induced tumorigenesis in ovariectomized animals was reciprocally related to the degree of protection afforded by that steroid in intact animals. That is, tumor incidence was approximately 20% in both ovariectomized and intact rats treated with E_2 while E_1 treatment produced a 55% incidence in both situations. This may indicate that tumor incidence reflects a balance between the tumorigenic and anti-tumorigenic pathways stimulated by these two estrogens. In standard biological and biochemical assays, E_2 is considered more potent than E_1 by virtue of its higher binding affinity with the estrogen receptor (43). Estrogens act in the cell by stimulating transcription of specific genes, the products of which regulate physiological pathways such as cell proliferation or apoptosis (44-46). It may be that pathways mediating anti-tumorigenic effects of estrogen are more efficiently stimulated by the stronger of the two hormones.

In summary, the ovariectomized, MNU treated rat serves as a model for testing the tumor promoter activity of hormones in the mammary gland. In this model system, E_1 was a more efficient tumor promoter than was E_2. However, full tumorigenesis was not achieved by estrogen stimulation alone; a synergy between E_1 and P_4 was required for tumorigenesis comparable to that seen in ovary-intact animals. These observations support the notion that

combined estrogen plus progestin therapy may pose a greater risk of breast cancer in postmenopausal women. On the other hand, the ovary-intact, MNU treated rat serves as a model for testing the anti-tumorigenic effects of hormone treatments. In this model, E_2 is more efficient than E_1 at reducing tumor incidence. It may be that tumorigenesis reflects a balance between the tumor promoter activity and the anti-tumorigenesis action of estrogens. Furthermore, it may be possible to identify selective estrogen receptor modulators (SERMs) that favor the anti-tumorigenic pathways.

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Table I. Summary of tumor formation and estrogenic endpoints in MNU-initiated, hormone-treated rats at 8 months after MNU treatment.

	Treatments[a]			
	Ovxd	+E2	+E1	Intact
Mammary Tumors				
animals w/ tumors:	0/20 (0%)[c]	5/21 (24%)[c]	6/11 (55%)[d]	10/10 (100%)
tumor latency (mos.)[b]:	-	4.8 ± 0.83	4.5 ± 0.55	3.4 ± 0.30
Estrogenic Effects				
uterine wt (mg):	132 ± 7.6[c]	445 ± 33.6[d]	504 ± 21.9[d]	610 ± 98.4
pituitary wt (mg):	13.5 ± 0.63	22.0 ± 2.69[cde]	16.0 ± 0.55[cd]	11.2 ± 1.95
body wt (g):	347 ± 6.4[c]	239 ± 5.2[cde]	300 ± 4.9[d]	298 ± 5.8

a. Ovxd, ovariectomized control; E2, estradiol; E1, estrone; Intact, ovary-intact control. Values are given as tumor incidence or as means ± SEM of other parameters.

b. time to first palpable tumor after MNU treatment

c. $P < 0.05$, vs. Intact

d. $P < 0.05$, vs. Ovxd

e. $P < 0.05$, vs. E1

FIGURE LEGENDS

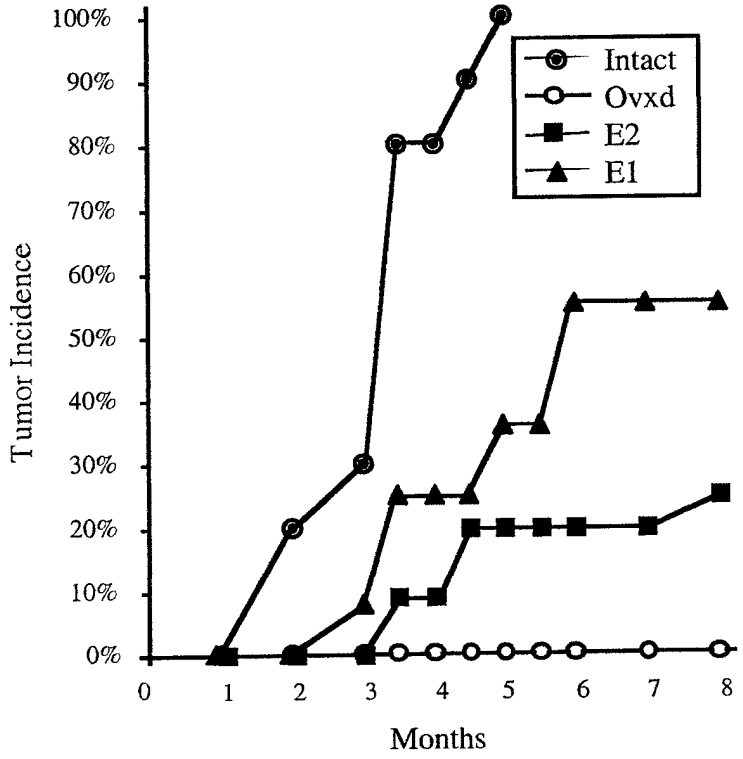
Fig. 1. Tumor incidence over time following MNU treatment. Ovariectomized rats were treated with a single injection of MNU and slow release capsules of estradiol (E2) or estrone (E1). Control animals received no hormone (Ovxd) or were ovary-intact (Intact). The percentage of animals bearing palpable mammary tumors was determined beginning one month after MNU injection.

Fig. 2. Effects of hormone treatments in ovariectomized and intact rats. MNU was injected and hormone treatment begun in ovariectomized or intact rats. Tumor incidence was determined at 8 months after MNU injection. The bars represent the percentage of animals with tumors; the numbers within the bars indicates the actual fraction of animals that had tumors. Statistical analysis: a, $P < 0.01$ vs. ovariectomized control (cont); b, $P < 0.01$ vs. intact control. There was a statistical difference ($P = 0.042$) between ovariectomized animals receiving estrone (E1) or progesterone plus estrone (P + E1).

Fig. 3. Mammary morphology: Effect of hormonal treatment in ovariectomized rats. Animals were ovariectomized and treated by subcutaneous implantation of Silastic capsules containing hormone and one month later the mammary glands were removed. Whole mount preparations were viewed at low (A, C, E) and high (B, D, F) power magnifications. Treatments were: no hormone control, A & B; estrone, C & D; progesterone plus estrone, E & F.

Fig. 4. Mammary morphology: Effect of hormonal treatment in ovary-intact rats. Animals were treated by subcutaneous implantation of Silastic capsules containing hormone

and one month later the mammary glands were removed. Whole mount preparations were viewed at low (A, C, E) and high (B, D, F) power magnifications. Treatments were: no hormone control, A & B; estrone, C & D; estradiol, E & F.



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Tumor Promoter Effects of
Estrogens and Progesterone....
Fig. /

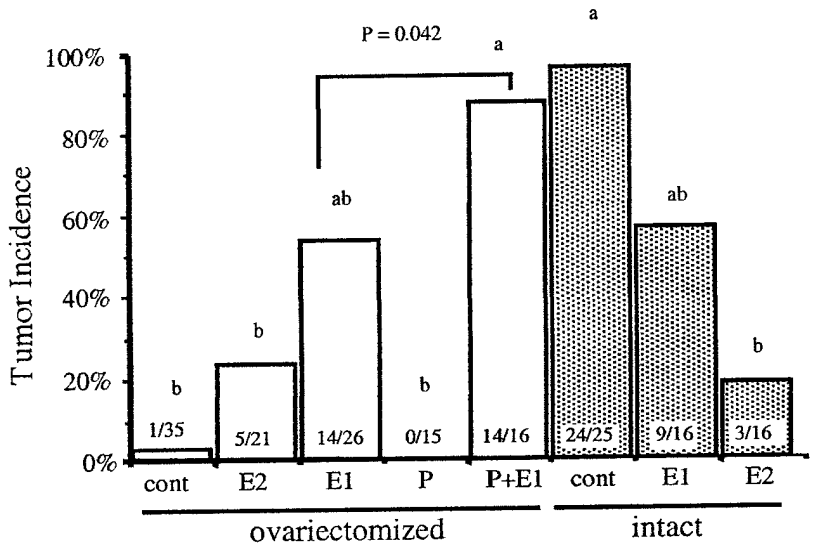

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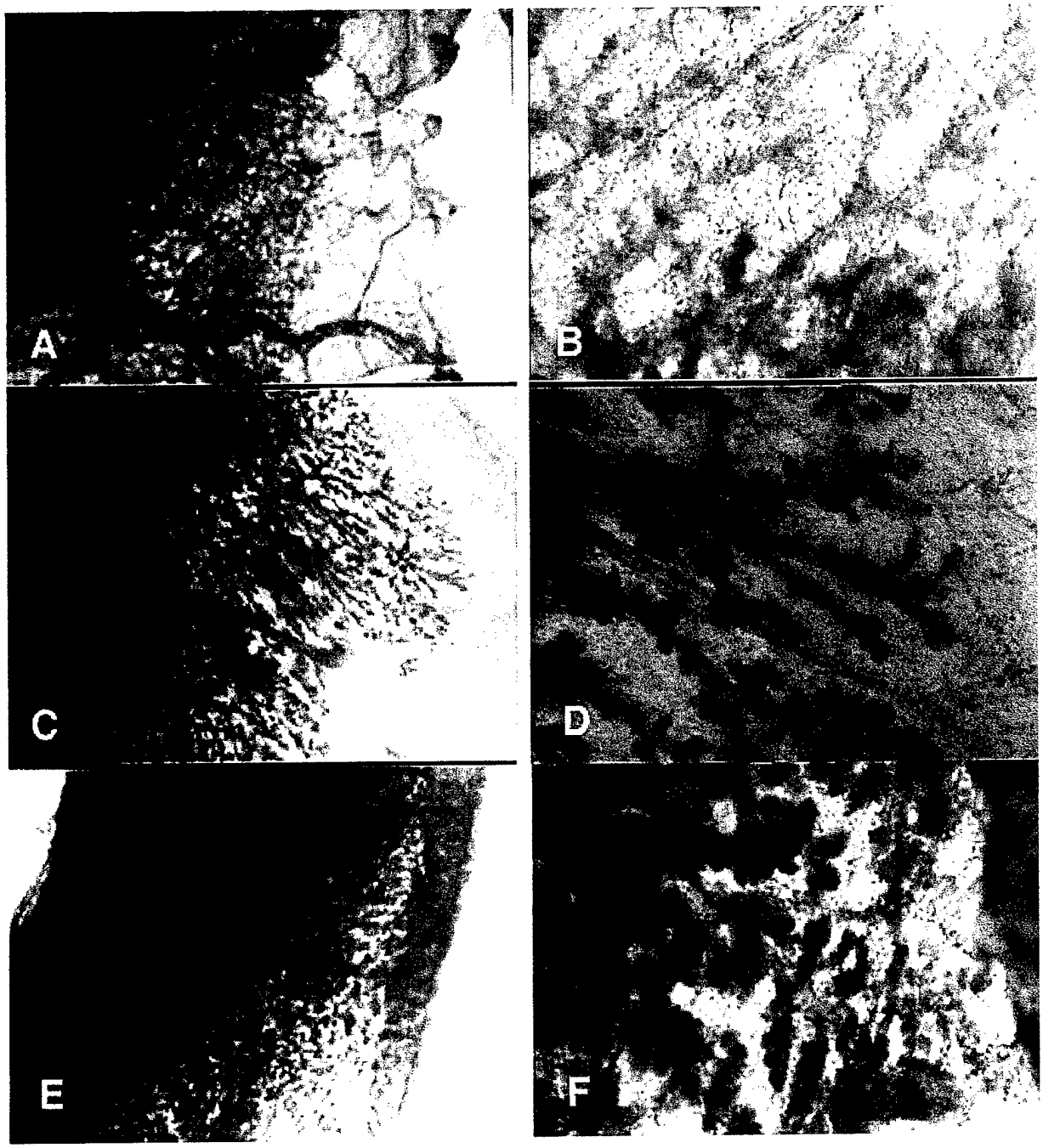
Fig. 2



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Estrogen and Progesterone....
Fig. 3

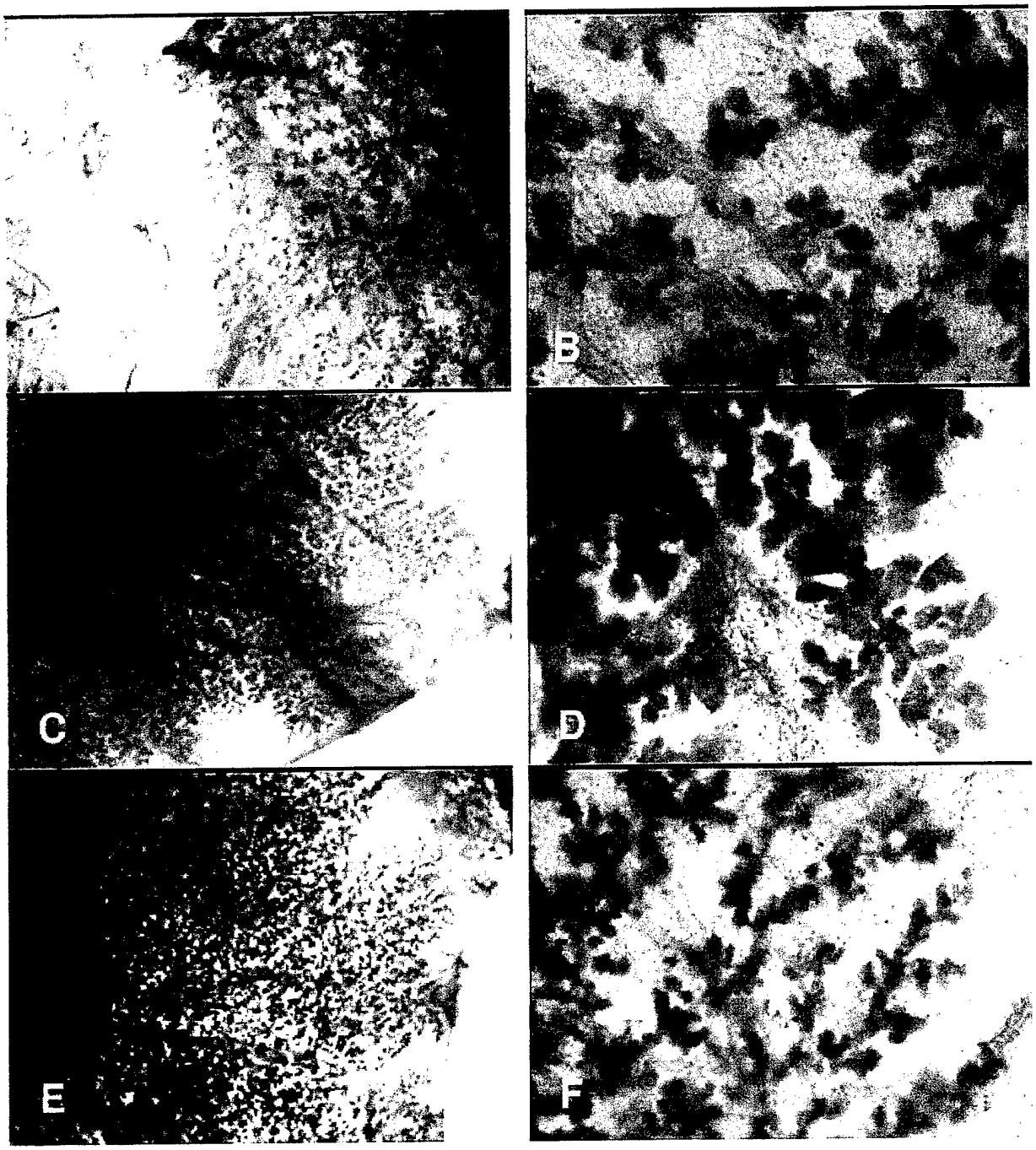

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Fig. 4